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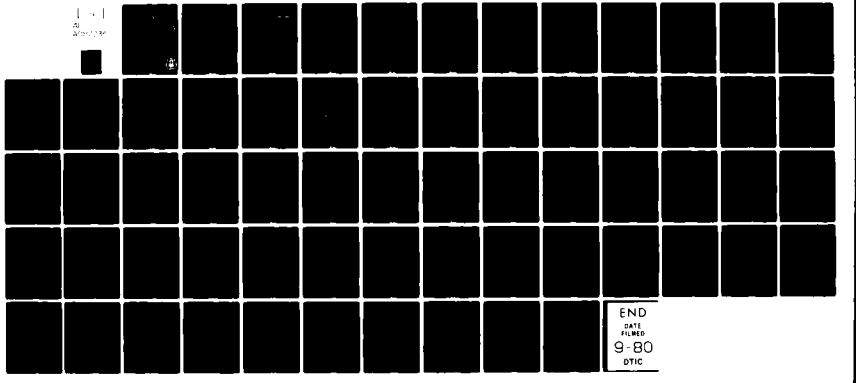

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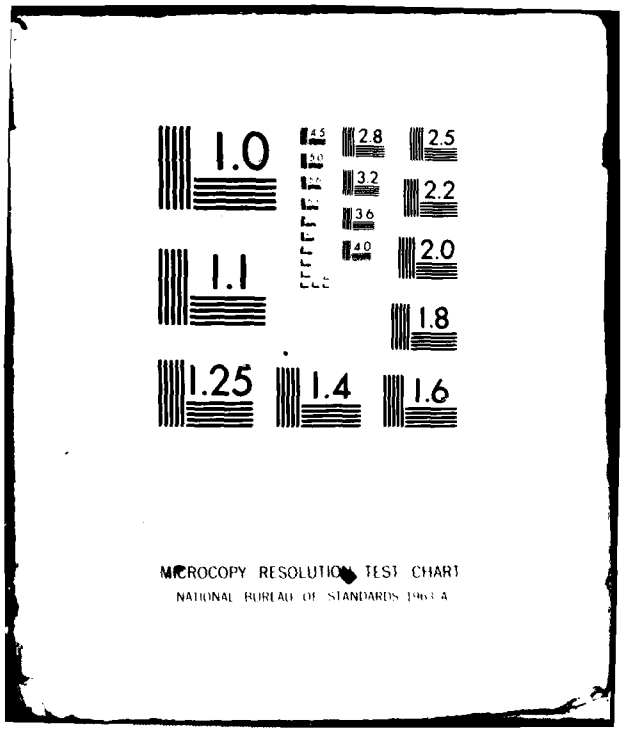
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A SHORT-TERM TOXICITY SCREENING TEST USING PHOTOBACTERIA -- A FEASIBILITY STUDY

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Beneckea harveyi	p-nitrobenzonitrile	3,4-dinitrotoluene
Bioluminescence	2,3-dinitrotoluene	2,3,6-trinitrotoluene
Photobacteria	2,4-dinitrotoluene	2,4,6-trinitrotoluene
Toxicity	2,5-dinitrotoluene	
m-nitrobenzonitrile	2,6-dinitrotoluene	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report describes a feasibility study using photobacterium Beneckea harveyi as the biologic system to test for toxicity. More specifically, the bioluminescent activity was used to assess the bacterial response to toxic chemicals. The chemicals used in this study are 2,3-dinitrotoluene, 2,4-dinitrotoluene, 2,5-dinitrotoluene, 2,6-dinitrotoluene, 3,4-dinitrotoluene 2,3,6-trinitrotoluene, 2,4,6-trinitrotoluene, p-nitrobenzonitrile and m-nitrobenzonitrile. Three dose-response studies were conducted with each of →

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20. Abstract (Continued)

the nine chemicals and an effective concentration which causes a 50% reduction in bioluminescence (EC50) was statistically computed. The mean of the three EC50 values were compared to the LD50 of rats and mice, the 96-hour LC50 for fathead minnows (Pimephales promelas), and the 48-hour EC50 for water flea (Daphnia magna). Of these comparisons, only the LD50 of male mice showed a fairly good degree of correspondence.

It is concluded that reproducible dose-response curves can be obtained from the photobacterial test system and it is recommended that further testing be conducted to increase the number of compounds tested for further comparisons with other species.

SUMMARY

A photobacterial test system was developed and the test procedure standardized. Dose-response studies were conducted using nine chemicals identified as environmental pollutants resulting from TNT production: 2,5-dinitrotoluene, 2,3,6-trinitrotoluene, *m*-nitrobenzonitrile, 3,4-dinitrotoluene, *p*-nitrobenzonitrile, 2,4,6-trinitrotoluene, 2,3-dinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. Estimates of the EC50 were computed for each of the nine chemicals. A comparison of the bacterial EC50 values and the toxic response in minnows (96-hour LC50) and daphnia (48-hour EC50) did not result in a high degree of correspondence. Thus, it was concluded that the photobacterial test system was not highly predictive of minnow or daphnia toxicity. A similar comparison with the acute 14-day LD50 values of the mouse and rat resulted in a fairly good degree of correspondence in the toxic response of male mice. Thus, further studies are recommended to expand the number of chemicals tested in both the photobacteria and male mice.

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INTRODUCTION

In response to federal regulations and internal requirements, the US Army conducts health hazard assessments of specific Army activities. Certain sources of chemical pollution that are potential health hazards to industrial workers, troops, or the environment have been identified:

1. the manufacture and demilitarization of propellants, explosives, smokes and obscurants, and chemical agents at industrial complexes,

2. the use of chemical smokes and obscurants during field training exercises,

3. the operation of weapon systems.

Aqueous discharges from munitions manufacturing can affect areas downstream of the production facility. The human population, indigenous wildlife, aquatic life, domestic animals, and crops may be exposed to the discharged pollutants or their degradation products. Workers at the production facility also risk exposure to these chemicals.

During training, troops are directly exposed to aerosolized chemicals from the field use of smokes and obscurants. Combustion products of propellants from rockets, artillery, and other weapon systems can also be a source of exposure to troops in training. The use of chemical smokes and obscurants in field training may cause environmental contamination, which poses additional toxicity problems.

Munitions that are never used are eventually disposed of in industrial "demilitarization" facilities. Workers at these facilities may be exposed to decomposition products that differ from the manufacturing by-products. The discharge of these decomposition products to the environment may pose toxicity problems different than those encountered at production facilities. For these reasons, research areas at the US Army Medical Bioengineering Research and Development Laboratory (USAMBRDL) include industrial worker exposures, troop exposures, and environmental pollution.

The number of chemicals discharged into the environment as pollutants or encountered in the workplace far exceeds the number that the Army can adequately assess for potential toxicity. The conventional series of toxicity tests is comprehensive, but the number of tests that may be reasonably conducted is limited by time and costs. A typical health hazard assessment study requires 3 to 5 years and costs $300,000 to $1,500,000 per compound. This strategy for health hazard assessment thus does not permit comprehensive evaluation of each chemical currently in use, or of the many new chemicals synthesized by industry, or of known chemicals proposed for new uses.

One of the solutions to this dilemma, proposed by Muul *et al.*[1] in a letter to *Science*, is the development of a battery of short-term toxicity tests screening that predict chronic toxic effects. The feasibility of

developing such tests (excluding mutagenesis/carcinogenesis assessment) is evaluated in studies by Shanahan[2] and Thomas.[3] Nardone[4] recently reviewed the *in vitro* toxicity tests currently in use or in the process of being validated. These publications emphasize the need to develop and validate short-term toxicity tests that predict chronic toxic effects.

In this project, the photobacterium was selected as the indicator organism for the development of a toxicity screening system. The biochemistry, physiology, and genetics of bacterial bioluminescence have been reviewed by a number of authors,[6-9] most recently by Hastings and Nealson.[5] Other reports[10-13] have also provided a detailed discussion of the taxonomic characterization of bioluminescent bacteria. A diverse spectrum of studies[14-19] has provided information on cellular control of synthesis of enzymes and cofactor requirements for bioluminescence, including studies on a variety of mutants. An extensive series of *in vitro* chemistry studies[20,22] has defined the stoichiometry and mechanism of *in vivo* light emission by photobacteria. The latter studies suggest that the chemical reactions responsible for *in vivo* light production are catalyzed by two enzyme systems:

$$H^+ + NADH + FMN \xrightarrow{\text{reductase}} NAD^+ + FMNH_2$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + h\nu$$

Nealson *et al.*[14] and McIlvaine and Langerman[23] have shown that a consistent pattern of growth and bioluminescence was observed when a fresh culture medium is seeded with bacteria. This pattern is characterized by an increase in the number of bacteria until a plateau in population density is reached. These authors suggest that the nutritive content of the medium probably limits the growth of the bacterial population in such a fixed volume of medium. The onset of bioluminescent activity lags behind the onset of the bacterial population increase by several hours. Bacterial bioluminescence increases to a peak followed by a decay until it approaches zero. The relationship between the increase in bacterial population size and the resultant peak in bioluminescent activity was used in this study. Since the bioluminescent peak is dependent upon bacterial replication and an intact "luciferin-luciferase" system, it is expected that any chemical that interferes with either aspect will result in a change in the bioluminescent activity of the bacterial population tested.

There are few reported investigations using bacterial bioluminescence to detect or quantitate the relative toxicity of different chemicals. Johnson *et al.*[24] studied the acute flash response when oxygen was provided to photobacteria grown under anaerobic conditions. Urethane tested at a 1 M concentration nearly abolished the flash. In concentrations as high as 0.01 M, cyanide had no effect on the flash. More recently, luminescent bacteria were used to detect air pollutants.[25-27] The RPC Corporation[28] developed a system using photobacteria to detect vapors of chemicals used in high explosives. A study with a similar goal but using an *in vitro* chemical approach was attempted by Goodson *et al.*[29] Bulich[30] used the

bacterial bioluminescence system as an acute water toxicity monitor. This test procedure reconstitutes lyophilized photobacteria in a buffered solution and compares light output before and after addition of the test chemical. Each of these tests is based on the acute response of the bioluminescence system to challenge by toxic substances.

The study in this report differs from previous studies in that the end point being measured (peak bioluminescent activity) is dependent on an intact metabolic system indicated by the increase in bacterial population density and an intact enzyme system producing luminescence.

OBJECTIVES

This report describes an attempt to assess the feasibility of using marine photobacteria in a short-term toxicity screening system. Changes in spontaneous bioluminescent activity of photobacteria induced by chemicals previously tested in higher organisms will be used to assess the system's predictive ability.

The specific objectives of this study are to:

1. select a liquid culture medium that sustains growth and the luminescent activity of photobacteria

2. select a strain of photobacteria

3. define the time-course of baseline bioluminescence

4. select chemicals for testing

5. conduct dose-response studies for each chemical

6. assess feasibility of the test system.

MATERIALS AND METHODS

Chemicals Tested. Nitrobenzonitriles and nitrotoluenes were selected for testing because they occur as water pollutants at Army munition production and packing locations, and thus pose potential environmental hazards. The wastewater discharged from such plants has been partially characterized. The toxicity of a number of the chemicals identified in the wastewater has been reported by Pearson et al.[31] for the daphnid and minnow and by Lee et al.[32] for the rat and mouse. The nine chemicals selected for testing were 2,5-dinitrotoluene, 3,4-dinitrotoluene, 2,3-dinitrotoluene, 2,4-dinitrotoluene, 2,6-dinitrotoluene, 2,3,6-trinitrotoluene, 2,4,6-trinitrotoluene, *p*-nitrobenzonitrile, and *m*-nitrobenzonitrile. The source, identity, and purity of the nine chemicals used in this study are described in a report by Spanggord et al.[33] The purity was greater than 99% for the above listed chemicals.

Photobacteria Tested. Nine different species or subspecies of bacteria were purchased from the American Type Culture Collection (Table 1). These bacteria were selected because of their ability to grow at or above room temperature. This characteristic eliminates the requirement for an incubator with refrigeration.

TABLE 1. BACTERIA SCREENED

Bacteria	ATCC No.[a]	Incubation Temperature (°C)[b]
Photobacterium pierantonii	14546	26
Photobacterium mandapamensis	27561	30
Photobacterium leiognathi	25521	26
Photobacterium leiognathi	25587	26
Photobacterium sepia	15709	26
Photobacterium phosphoreum	11040	18
Lucibacterium harveyi (Beneckea harveyi)	14126	26
Vibrio fischeri (Achromobacter fischeri)	7744	26
Vibrio fischeri (Photobacterium fischeri)	25918	26

a. American Type Culture Collection identification number.
b. Incubation temperature recommended by ATCC.

Preparation of the Culture Medium. Dehydrated Bacto Photobacterium Broth* was purchased from Difco. The working solution used in photobacterial cultures was made according to the directions provided by Difco. To sterilize the medium, the mixture was autoclaved in a tightly capped KIMAX bottle for 15 minutes at 121°C and 14 psig and permitted to cool to room temperature. Because a substantial amount of the solute remained undissolved, the solution was decanted into sterile plastic flasks for storage and subsequent use. This solution, referred to as the Difco culture medium, was used for all studies except the initial screening of the bacteria, when the Difco medium was not available. The medium that was used consisted of the ingredients listed in Table 2. This medium was filter sterilized using a 0.2-micron Nucleopore filter; it is referred to as the USAMBRDL culture medium.

* Mention of a proprietary product is for identification purposes only and does not imply endorsement by the Department of Army or Department of Defense.

TABLE 2. USAMBRDL CULTURE MEDIUM

Item	Amount
NaCl	30.0 g
Na_2HPO_4	14.0 g
KH_2PO_4	2.0 g
$(NH_4)_2$ HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Bacto yeast extract	5.0 g
Tryptone	5.0 g
Peptone	5.0 g
Glycerol	1 mL
Add distilled water to 1 liter	

Photobacterial Culture Maintenance. The stock culture of photobacteria was maintained by transferring 1 µL of a photobacterial suspension from a 24-hour culture into 7 mL of fresh Difco or USAMBRDL culture medium once every 24 hours. This photobacterial suspension is referred to as the stock culture.

Preparation of Photobacterial Test Culture. Preparation of the test culture involved adding enough 24-hour photobacterial stock culture to fresh medium to achieve a final suspension with an optical density reading of 0.046. All measurements of optical density were made using a Spectronic 20 set at a wavelength of 620 nm. This suspension is referred to as the photobacterial test culture.

Preparation of the Salt Solution. The salt solution consisted of the first five chemicals listed in Table 2 and distilled water. Thus, the carbon-containing ingredients were omitted. The solution was sterilized by autoclaving and is referred to as the salt solution.

Preparation of the Test Solutions. Each chemical to be tested was first dissolved in HPLC grade acetone, and varying amounts of this solution were added to the salt solution. The final concentration of acetone was adjusted to 10% by adding pure acetone. The final solution containing the test chemical and 10% acetone (v:v) is referred to as the test solution.

Preparation of the Control Solution. The solution used as a control in these studies consisted of 10% acetone in the salt solution and is referred to as the control solution.

Test Procedure. The test involved dispensing 10 µL of the test solution into glass test tubes (7 mm x 50 mm) in replicates of 6 for bioluminescence measurements at each concentration of a given test chemical. A group of 6 test tubes containing 10 µL of the control solution was prepared as the control group. A control group accompanied each different chemical tested. One hundred µL of the photobacterial test culture were added to each tube containing 10 µL of the test or control solution. Thus, the final volume of each test tube used for bioluminescence measurements was 110 µL. All test tubes were then incubated at 27° ± 0.3°C until the bioluminescent activity of the control cultures reached a peak. The luminescent activity of each culture was then measured and recorded. Luminescence was expressed in relative units, and the photometer was calibrated by a vial containing 1 µCi of carbon-14.

Chemical Analysis of Test Solutions

General Procedure for Test Chemical Analysis

All standards were prepared by dilution of known standard samples. An aliquot was pipetted and brought up to volume with acetone in a volumetric flask. Each series of standards contained at least three data points for the preparation of a standard curve, with all the samples falling between the highest and lowest points on the curve. Each standard was run at least three times and the areas averaged when estimating the standard curve.

A 2-mL aliquot of aqueous sample was placed in a screw cap vial, and 2 mL of methylene chloride were added. The vials were sealed and shaken, and a sample of the methylene chloride was injected into the gas chromatograph. The injection was repeated at least three times, and the results averaged. Extraction efficiency was assayed by extracting known aqueous solutions with methylene chloride and comparing the known concentration with that assayed by gas chromatographic analysis. All final concentrations reported herein were corrected for extraction efficiency.

Gas Chromatograph Conditions

Column:	Carbopack C/0.3% Carbowax 20 M/0.1% H_3PO_4
Initial Temperature:	150°C
Temperature Initial Hold:	5 minutes
Rate of Increase:	5.00°C/min
Final Temperature:	165°C
Final Temperature Hold:	4 minutes
Injection Temperature:	225°C

Flame Ionization Detector Temperature:	225°C
Attenuation:	2^3
Helium Flow:	30 mL/min

This analytical procedure was used to measure the amount of the test substances in the test solutions. No attempt was made to quantitate the final concentration of the test chemical after mixing the test solutions with the photobacterial test culture because of the small test volumes (110 µL) used in this study.

RESULTS

Selection of a Photobacterial Strain

Nine different species or subspecies of bacteria were purchased from the American Type Culture Collection. Each was rehydrated in the USAMBRDL culture medium and subcultured every 24 hours for 3 consecutive days. This was the only study conducted in a walk-in environmental chamber. The temperature was maintained at 20.5° ± 0.9°C and relative humidity at 95 ± 4%. Throughout the study, light was maintained from 0400 to 2000 hours, and darkness from 2000 to 0400 hours. Measurements of bioluminescence were made on three replicate samples transferred from this starting solution to 7 x 50 mm test tubes. A separate aliquot of the same photobacterial culture was placed in a Spectronic 20 cuvette and turbimetric measurements for bacterial population growth were made.

Of the bacteria tested, Vibrio fischeri (No. 7744) did not replicate in the culture medium used. Three other cultures did not produce any measurable bioluminescent activity during the study span. These were Photobacterium leiognathi (No. 25521 and No. 25587) and Photobacterium sepia. Cultures of Photobacterium pierantonii and Vibrio fischeri (No. 25918) showed a peak in bioluminescence after approximately 33 and 28 hours, respectively. Photobacterium phosphoreum produced a relatively constant level of bioluminescence with no clear peak. Cultures containing Photobacterium mandapamensis (Fig. 1) and Lucibacterium (Beneckea) harveyi (Fig. 2) resulted in a peak in bioluminescence after approximately 6 and 11 hours, respectively. Of the two species, Lucibacterium harveyi produced light at a greater intensity at peak bioluminescent activity.

For each of the species that demonstrated a peak in bioluminescence, the peak was preceded by an increase in population density, which was indicated by an increase in optical density of the bacterial culture as a function of time. Figures 1 and 2 are representative of the changes observed in optical density, and a comparison with the bioluminescence curve shows an initial increase in optical density with no detectable bioluminescent activity. The bacterial population growth seems to reach a plateau when a clear increase in bioluminescence is observable.

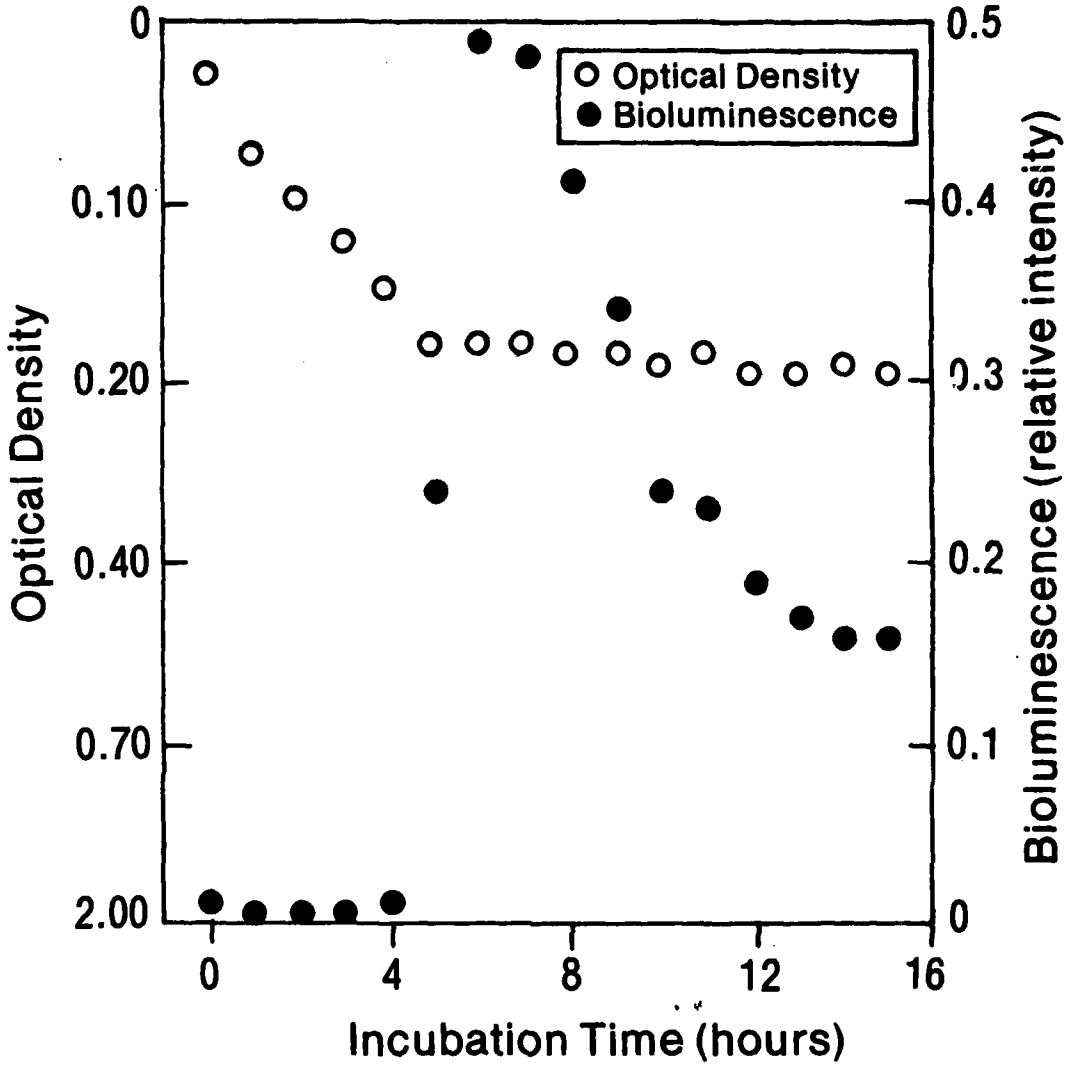

Figure 1. Population Growth and Bioluminescent Activity of Photobacterium mandapamensis.

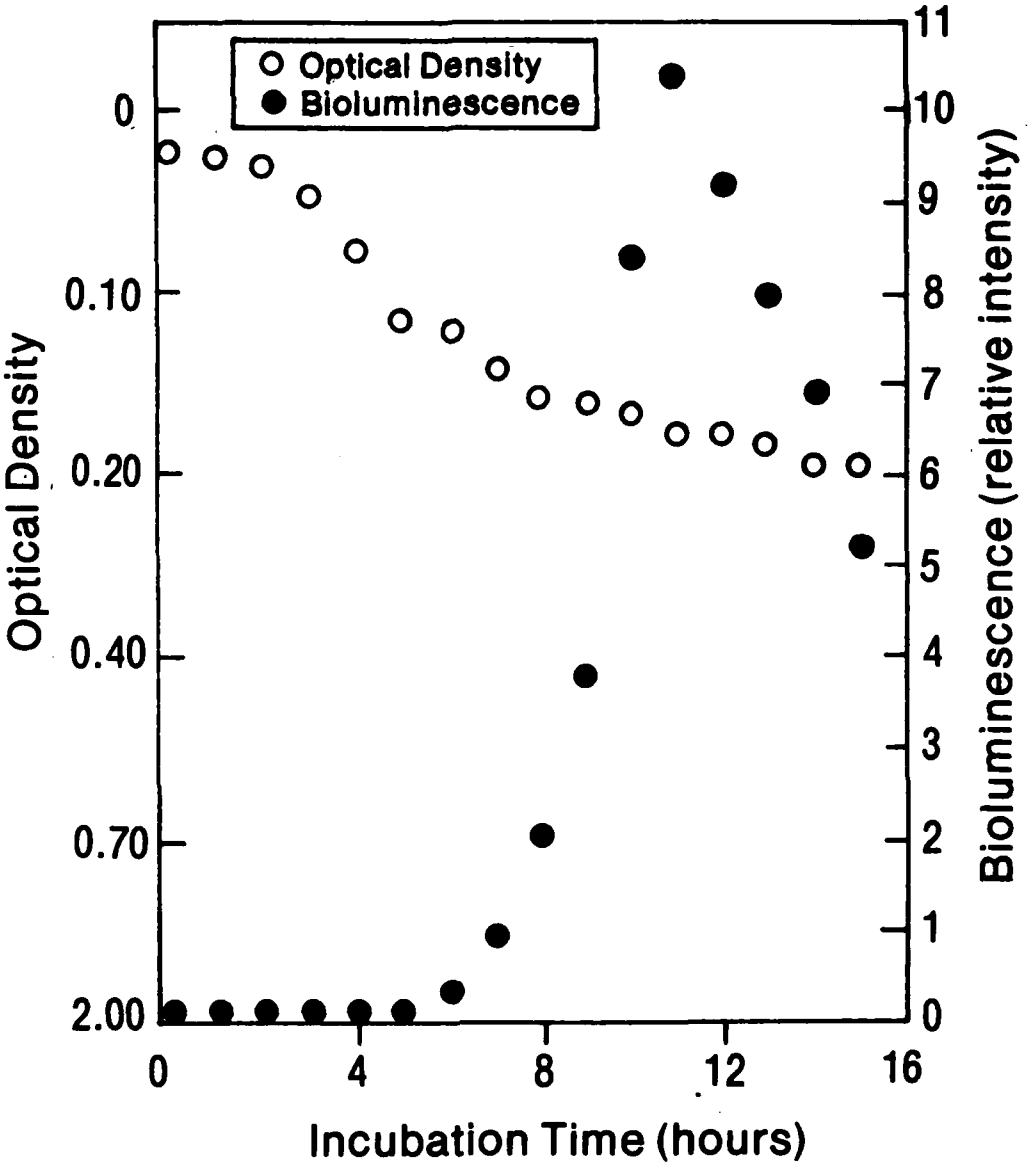

Figure 2. Population Growth and Bioluminescent Activity of Lucibacterium (Beneckea) harveyi.

Selection of Test Volume

To determine whether starting with different numbers of bacteria would result in different levels of luminescence, six different starting volumes were tested. Six replicates each of 20-, 40-, 80-, 100-, 150-, and 200 µL suspensions were used and the bioluminescence measured hourly until the last 2.5 hours of the study when 30-minute measurement intervals were used to more accurately define the peaks. The results, using the mean values of the six replicates, are shown in Figure 3.

The photobacterial cultures showed a phasic response in luminescence. The photometer was set at the least sensitive setting throughout the study, and a peak in luminescence within the maximal range of the photometer was observed for the 20-, 40-, 80-, and 100-µL starting volumes. Cultures with 150-µL and 200-µL starting volumes produced bioluminescent activity beyond the limits of the photometer's maximum range. The peak in luminescence occurred later as the starting volumes were increased, and the rate of increase in bioluminescence also seemed to be greater with larger starting bacterial number. The result of greatest interest was the difference in the height of the peak in bioluminescence with different starting bacterial number since this parameter was to be used in selecting the test volume for all subsequent studies.

Determination of Baseline Bioluminescence

To accurately plot the bioluminescent activity of a photobacterial culture as a function of time, a single test tube containing 100 µL of the photobacterial test suspension was placed in the photometer, and the photometer output was continuously recorded by a strip chart recorder. The results are shown in Figure 4.

The pattern of luminescent activity was characterized by three distinct phases. The first phase consisted of a slight decrease to little or no luminescent activity (lag phase); the second phase of a rapid and continuous increase to a peak in luminescent activity; and the third phase of a decrease from the peak toward the original level of luminescence with no subsequent increase during the study span.

A continuous recording of the incubator temperature is shown near the top of Figure 4. The temperature range was 26.8° to 27.2°C.

Effect of Acetone

Salt solutions containing 0, 5, 10, 15, and 20 percent (by volume) acetone were tested. Six replicates of each concentration were measured hourly for the first 7 hours and at 30-minute intervals thereafter for a total study span of 9.5 hours. A plot of these data is shown in Figure 5. No significant amount of bioluminescence was observed for the first 3 hours. Thereafter, all cultures displayed an increase in luminescent activity until a peak was observed after approximately 9 hours of incubation.

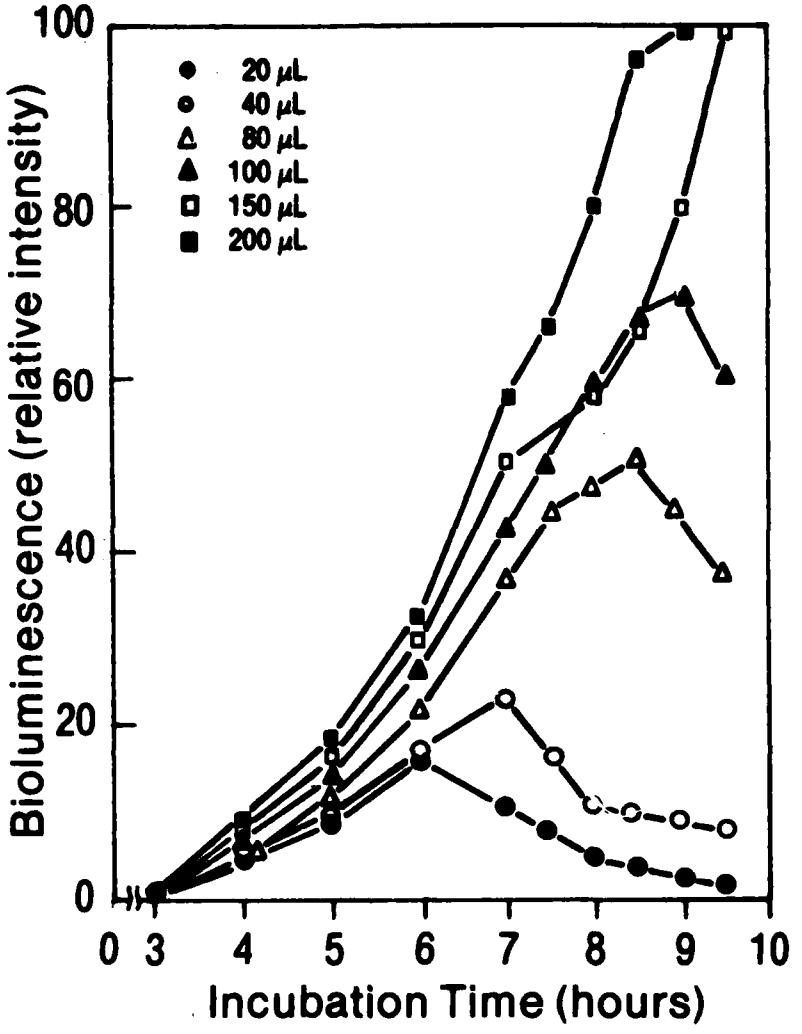

Figure 3. Bioluminescent Patterns of Beneckea harveyi Cultures with Different Volumes.

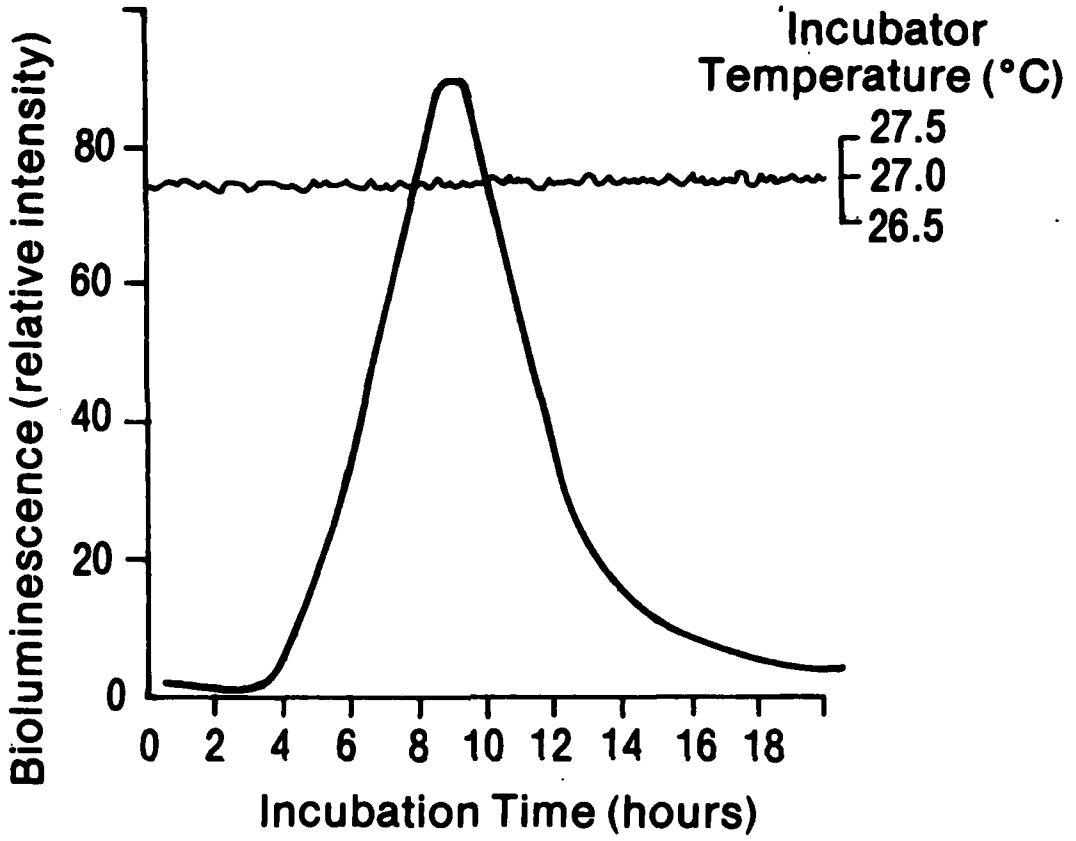

Figure 4. Baseline Bioluminescent Activity of Beneckea harveyi.

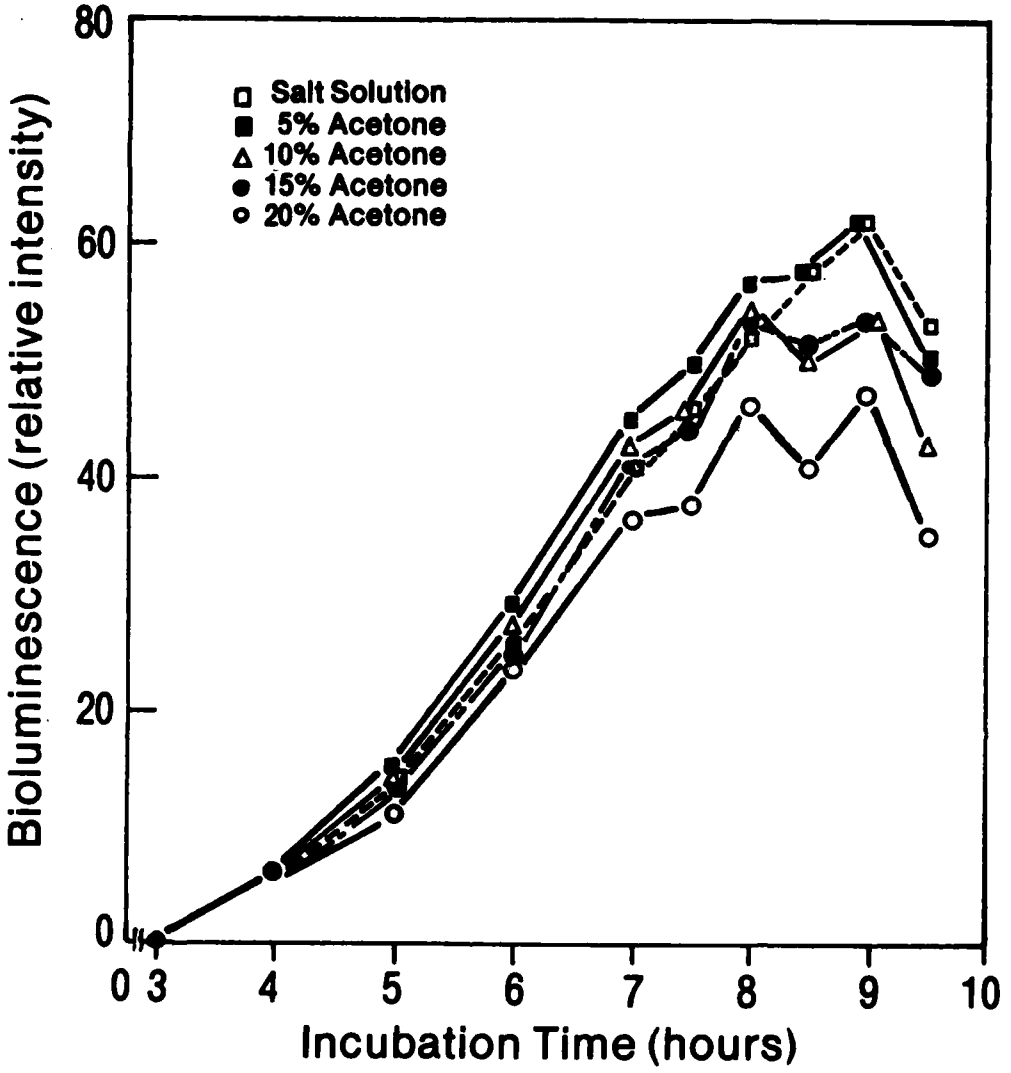

Figure 5. Effect of Acetone on Bioluminescence of Beneckea harveyi.

To determine whether there were any statistically significant changes in luminescent activity from different concentrations of acetone in comparison to the control (salt solution only) group, the Neuman-Keuls[34] multiple comparison test was performed on measurements taken at hour 4 through the end of the study. Hour 4 was selected because no measurable bioluminescence occurred prior to that point of the study. Statistical analysis of the data revealed no differences among the four groups until after 7 hours of incubation. At 7.5 and 8 hours of incubation, the 20% acetone group was significantly different at the $p = 0.05$ level from all others, and no differences were detected among the salt solution control, 5%, 10%, and 15% groups. For the last three time points (8 to 10 hr), three different levels of response to acetone were detected. There was a statistically significant ($p = 0.05$) difference between the salt solution and the 5% acetone solution. Similarly, the 10% and 15% solutions formed a second group that was significantly different from the first. The 20% acetone solution produced the greatest degree of response and was significantly different from the other two groups.

Dose-Response Studies

Preliminary studies were conducted to determine the concentration range that produces a partial response in bioluminescence. Dose-response studies were then conducted to determine the concentration of each chemical that reduced the peak in luminescent activity by 50% when compared to control groups. This concentration is referred to as the median effective concentration (EC50).

Three dose-response studies were conducted on each chemical. In the first study, five replicates were used for each concentration. In the second and third studies, six replicates were used. The same test solutions were used for all three studies.

There were no observed stimulatory effects on the bioluminescent activity of any of the chemicals tested. In every study, higher concentrations of the test chemical resulted in a reduction of bioluminescent activity. When these raw bioluminescence data were plotted as a function of increasing concentration, the resultant curve was a logarithmic function with a negative slope. Since the total range of the photometer was preset at 0 to 100 units, the raw data were converted using the following equation:

$$\text{Converted bioluminescence} = 100 - \frac{\text{raw data}}{\bar{x} \text{ of control group}} \times 100$$

The raw data were expressed as a percentage of the control group mean and by subtracting this value from 100, the bioluminescence readings increased as the concentration of the test chemical increased. Thus, the resultant slope was positive.

For each dose-response curve, the mean of the converted values were computed for each concentration tested. The means from each study (A, B, and C) were plotted on semi-log graph paper with logarithms of concentration (Figs. 6 through 14). The selection of the data-domain used to estimate the EC50 was based on a determination of the linear portion of the dose-response curve by visual inspection. The EC50 was estimated using a computer program developed at this Laboratory. The mean of the three EC50 values (Studies A, B, and C) was computed for each chemical and used for comparisons of potency among the chemicals tested as well as comparison to aquatic and mammalian toxicity data.

DISCUSSION

Establishing Test Parameters

Selection of a Photobacterial Strain. The criteria for selecting a final test species were that it must be able to grow in a liquid medium and produce a peak in bioluminescence within 12 hours of incubation. Should more than one bacterial species meet both criteria, the species producing the greatest amount of luminescence would be selected. Nine species or subspecies were preselected as potential candidates for the development of this test system.

The culture medium did not support growth of Vibrio fischeri (ATCC No. 7744) and thus no further testing was performed. Two subspecies of Photobacterium leiognathi (ATCC Nos. 25521 and 25587), and the species Photobacterium sepia (ATCC No. 15709) were eliminated because they did not produce any measurable bioluminescent activity. Photobacterium phosphoreum (ATCC No. 11040) was also eliminated because no definitive peak in bioluminescent activity was observed. Photobacterium pierantonii (ATCC No. 14546) and Vibrio fischeri (ATCC No. 25918) both produced a peak in bioluminescence but the peaks occurred after approximately 34 and 28 hours of incubation, respectively. These groups were eliminated because the time-to-peak after incubation was considered too long. Photobacterium mandapamensis (ATCC No. 27561) and Lucibacterium harveyi (ATCC No. 14126) both produced peak bioluminescent activity within 12 hours of incubation. The latter species was chosen for further development because it produced a greater intensity of bioluminescence at peak activity. These studies were conducted using the USAMBRDL culture medium. All subsequent studies were conducted using the Difco culture medium.

Standardization of Age of the Bacterial Test Culture. The age of the stock bacterial culture from which photobacterial test cultures are made may influence baseline bioluminescence and, consequently, the results of any challenge by external stimuli such as test chemicals. Since this project is a feasibility study with limited goals, it was decided to standardize the age of the starting culture so that it was no longer a variable rather than to attempt to determine whether this factor influenced baseline bioluminescence. Thus, the stock population was subcultured at 0730 each

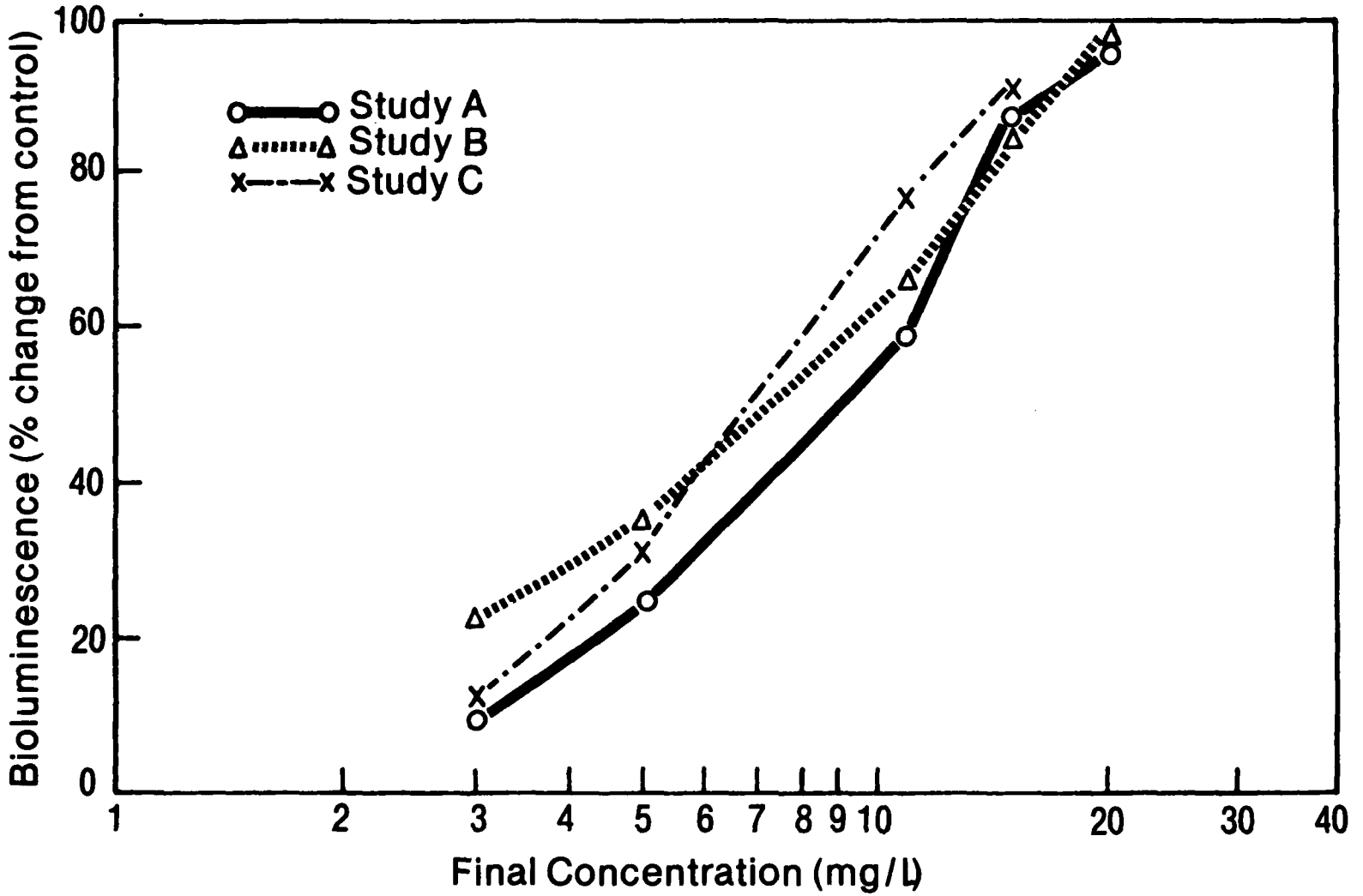

Figure 6. Response of Beneckea harveyi to 2,3-Dinitrotoluene.

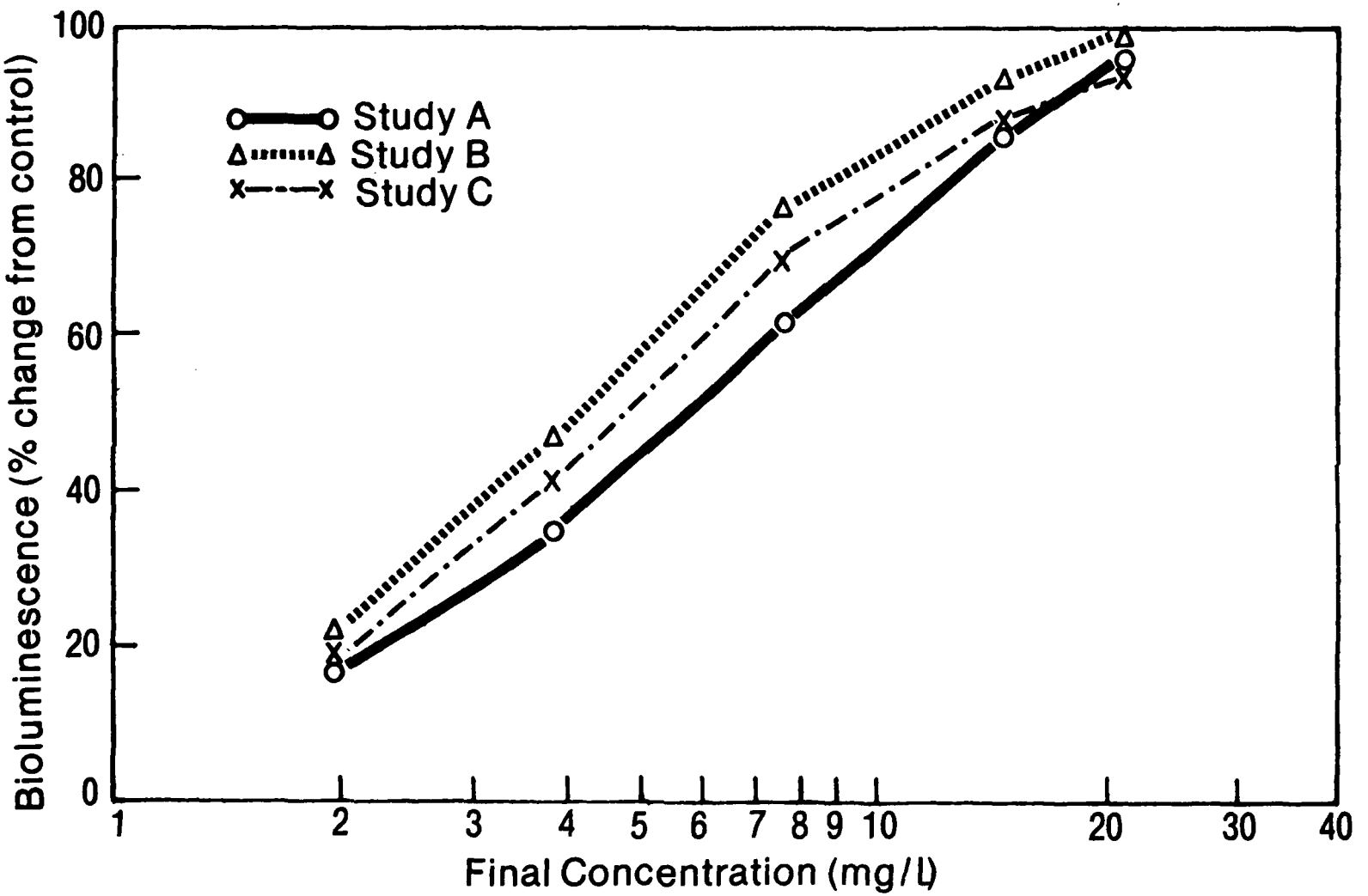

Figure 7. Response of Beneckea harveyi to p-Nitrobenzonitrile.

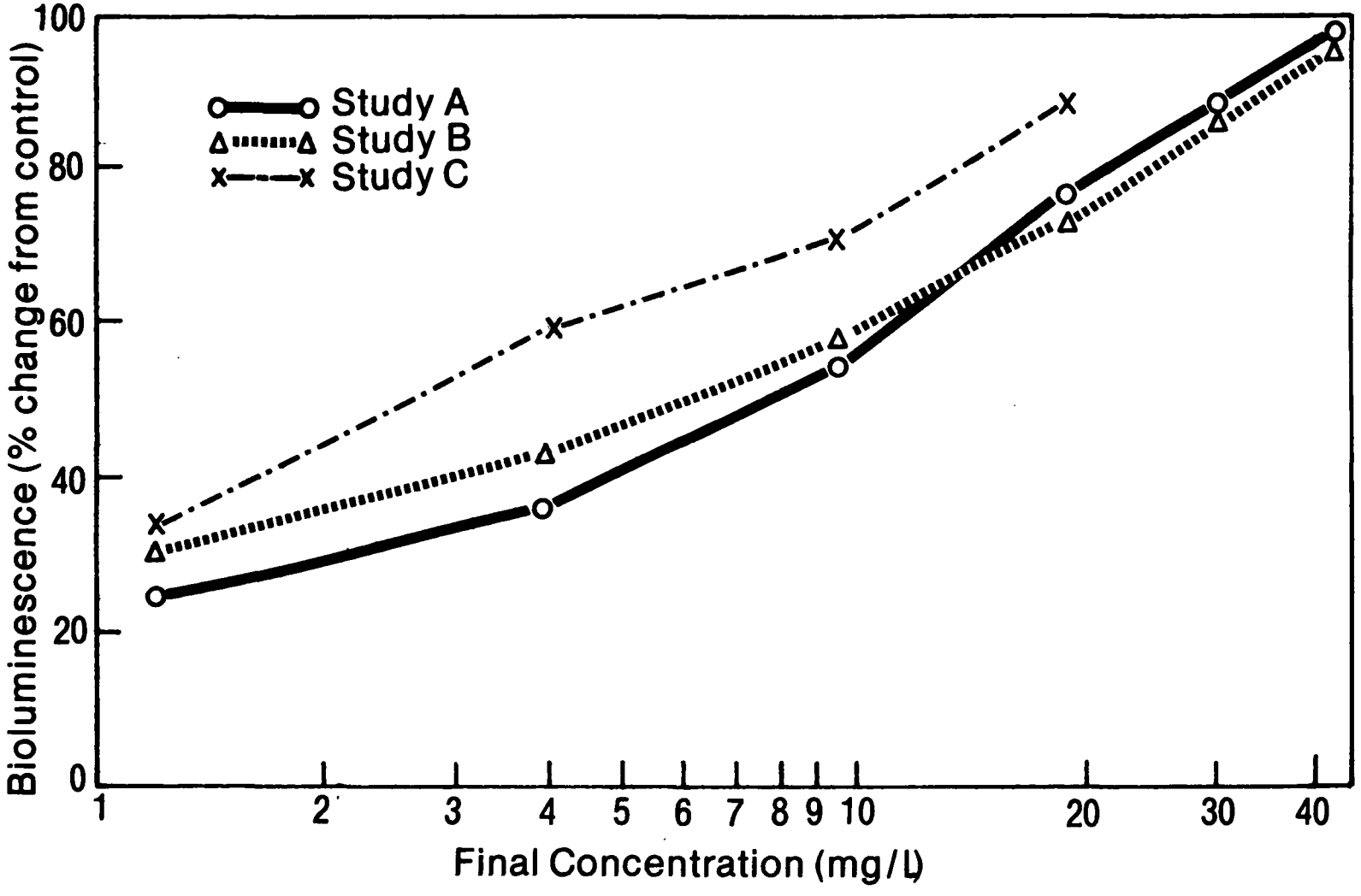

Figure 8. Response of Beneckea harveyi to m-Nitrobenzonitrile.

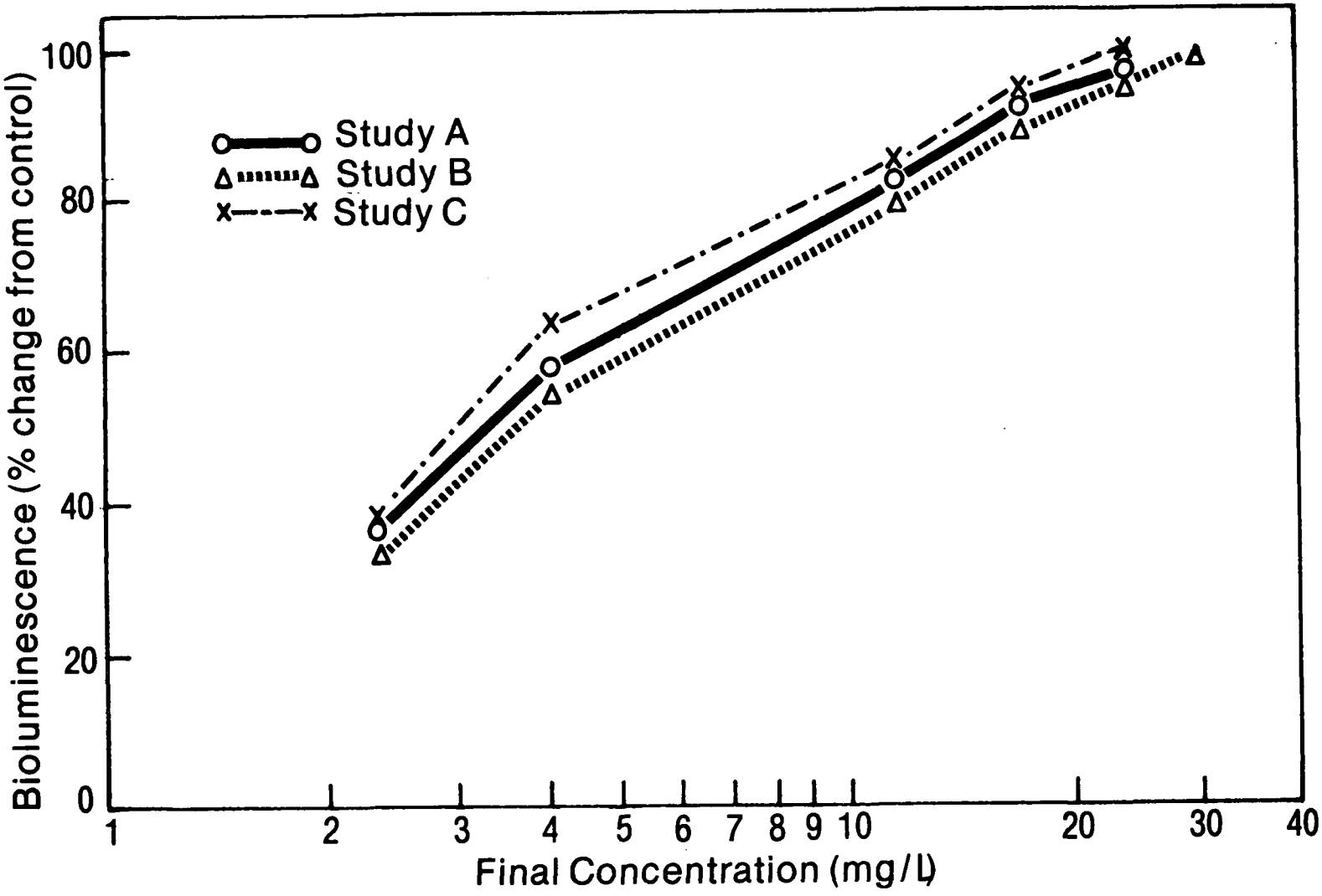

Figure 9. Response of Beneckea harveyi to 2,5-Dinitrotoluene.

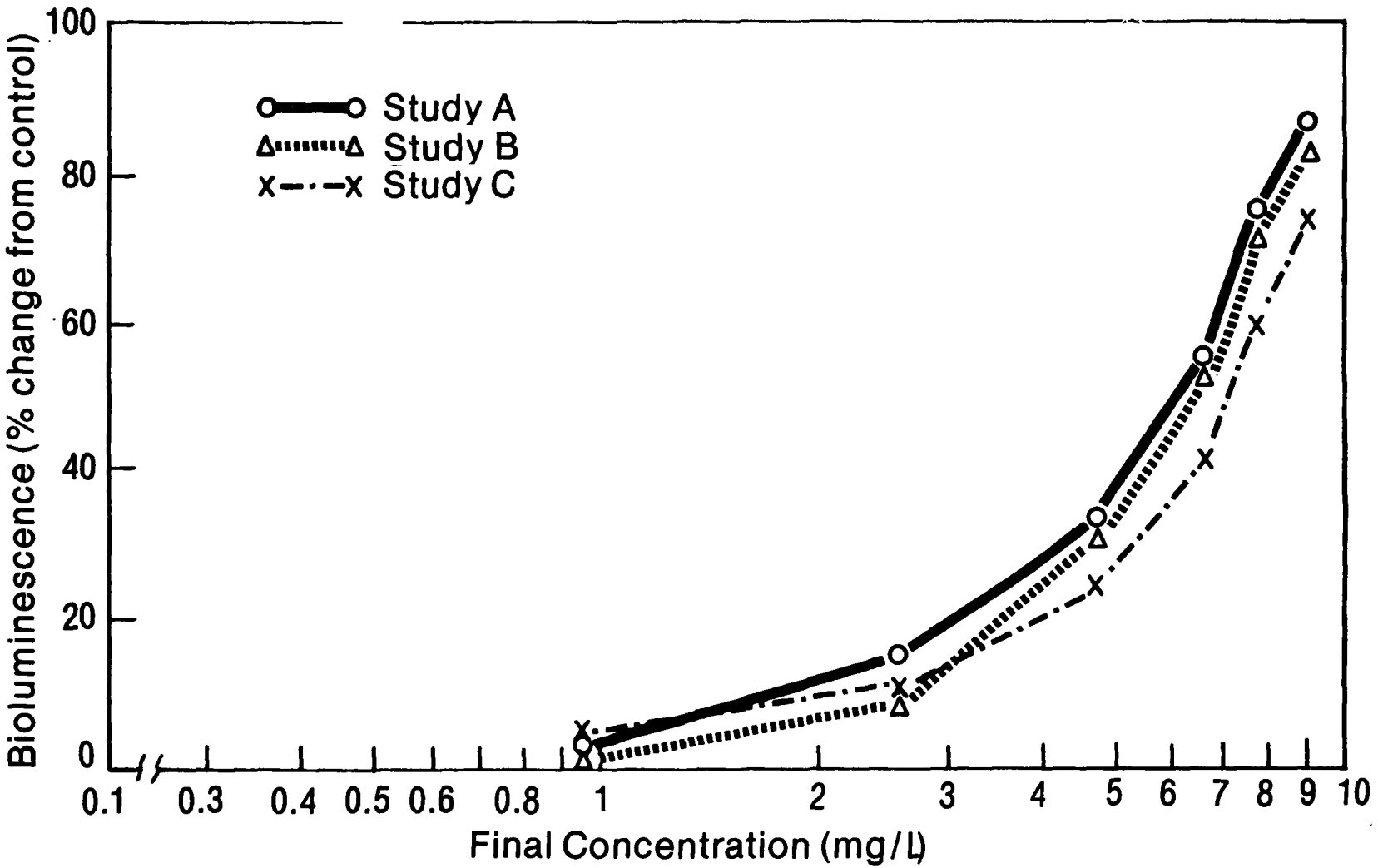

Figure 10. Response of Beneckea harveyi to 2,4,6-Trinitrotoluene.

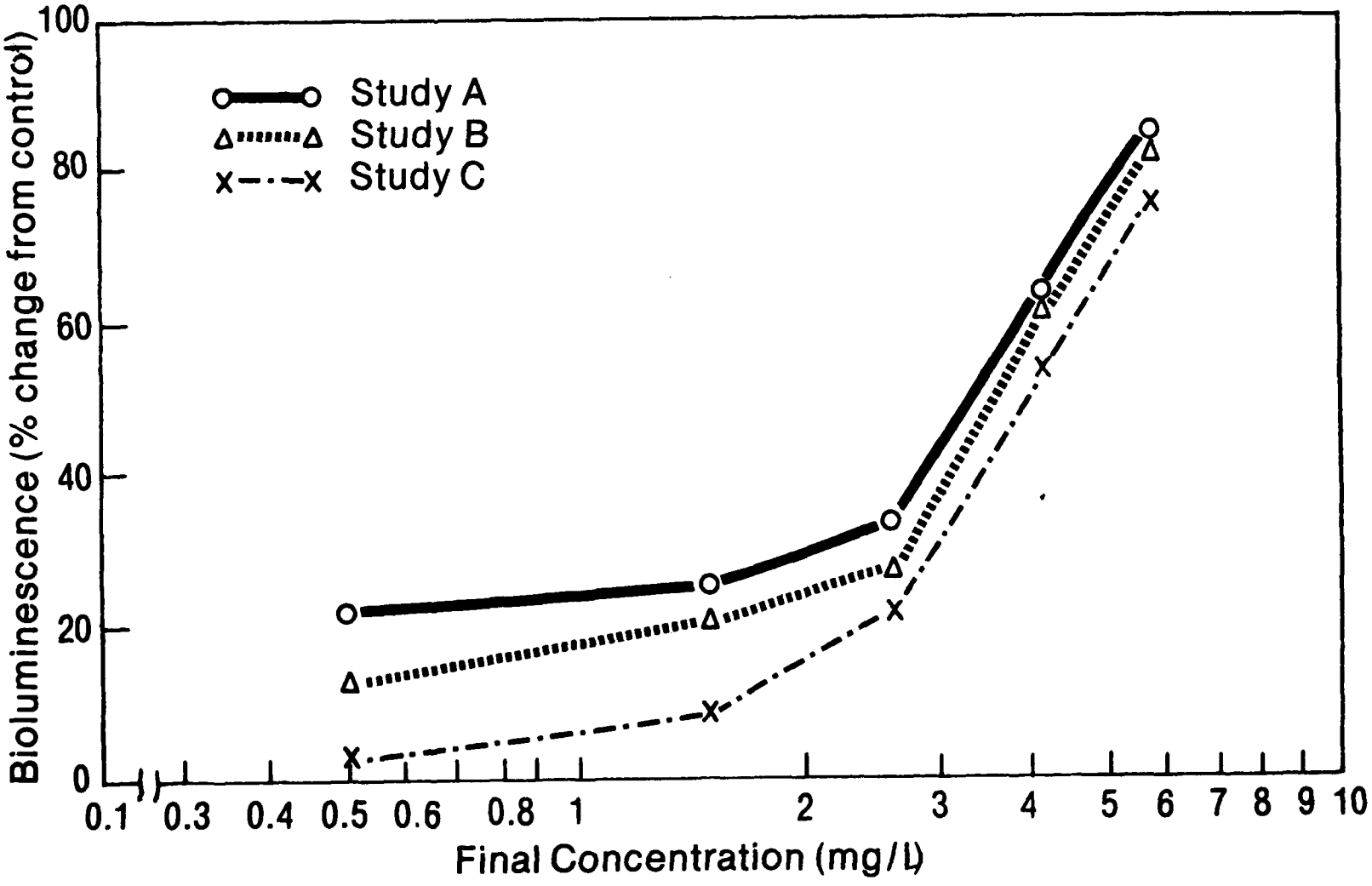

Figure 11. Response of Beneckea harveyi to 2,3,6-Trinitrotoluene.

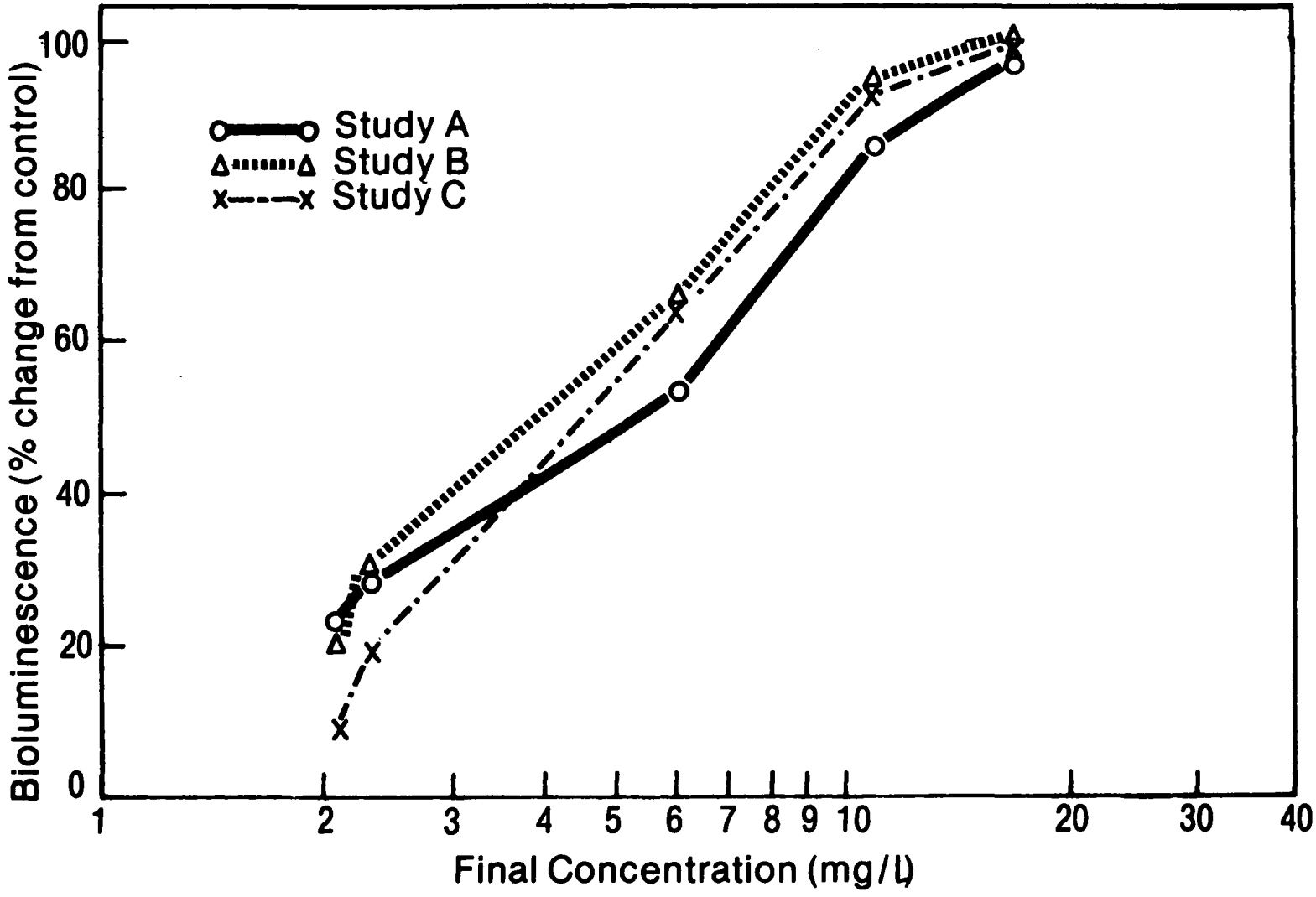

Figure 12. Response of Beneckea harveyi to 3,4-Dinitrotoluene.

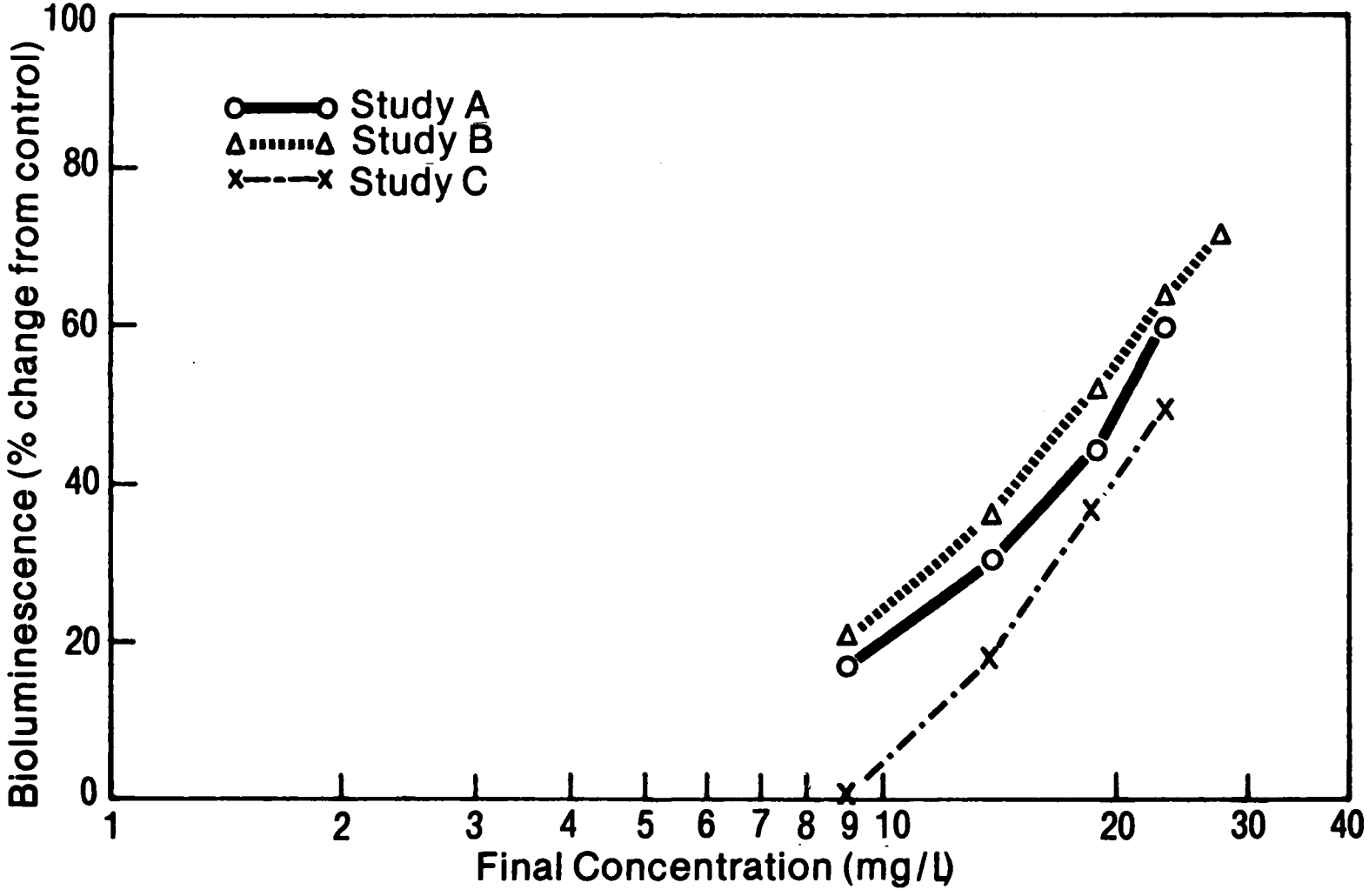

Figure 13. Response of Beneckea harveyi to 2,6-Dinitrotoluene.

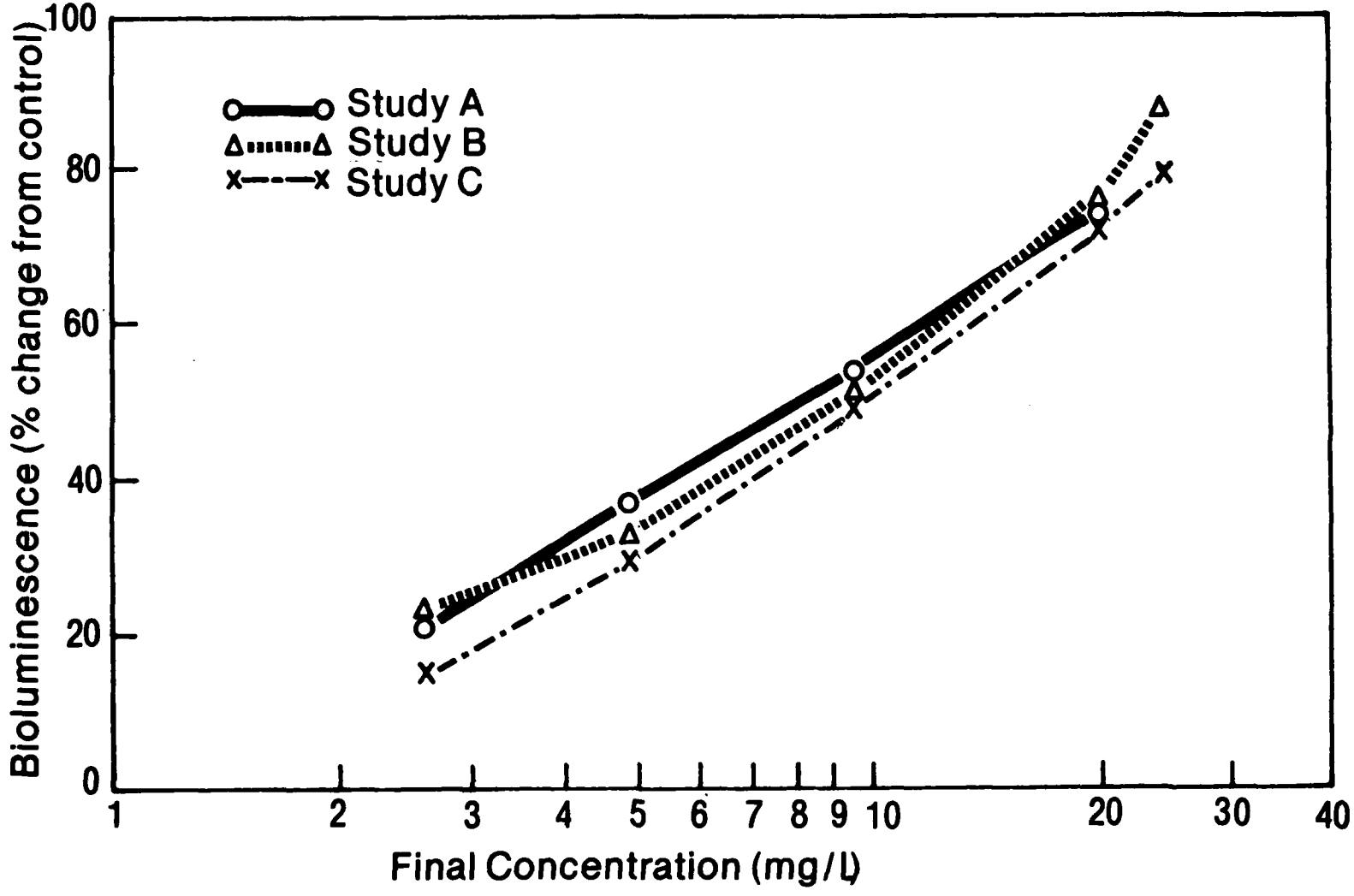

Figure 14. Response of Beneckea harveyi to 2,4-Dinitrotoluene.

day, and the photobacterial test cultures were also made from 24-hour-old stock cultures. The procedures described in the materials and methods section were followed to continue the stock cultures as well as to make the test cultures. Thus, any influence of the age of the bacterial culture was controlled by fixing the time and method of subculturing.

Selection of Test Volume. After selection of a strain of photobacterium, it was necessary to determine whether different starting volumes would result in different levels of peak bioluminescence. An optimal level of bioluminescence peaks between 50 and 100 units of relative intensity on the photometer. Six different volumes (20, 40, 80, 100, 150, and 200 µL), representing aliquots from the same starting mixture with an optical density of 0.046, were tested. The results (Fig. 3) clearly demonstrate that a starting volume of 100 µL results in a peak of approximately 70 units, which falls within a convenient working range of the photometer. This intensity of peak bioluminescent activity in the control group would permit observations of increases and decreases in bioluminescence resulting from test chemicals. Thus, all baseline bioluminescence and dose-response studies were conducted using 100 µL of the photobacterial test cultures.

Baseline Bioluminescence. A single tube of photobacteria was continuously monitored to establish a detailed time record of the course of bioluminescent activity (Fig. 4). A single peak was observed approximately 10 hours after incubation. The time to peak decreased in subsequent studies, and peak bioluminescence under similar culture conditions was observed after approximately 6 hours of incubation. The bioluminescent dynamics remained stable throughout the remainder of the studies. The change in shape of the bioluminescence curve may have resulted from a shift in the makeup of the stock photobacterial population. The nutritive properties of the Difco culture medium and the incubation conditions may have differed from those used by American Type Culture Collection. These factors may have induced multiplication of a selective segment of the original population, which had the inherent ability to grow faster, thus causing an earlier peak in bioluminescence. The typical bioluminescent curve for R. harveyi, starting with a lag phase described by Nealson et al.[14] as a period of conditioning of the medium by the growing cells, has been previously reported by McIlvaine and Langerman.[23] The greater level of bioluminescence and the earlier occurrence of a peak (compare Figure 4 and Figure 2) may be a result of a more nutritive medium (Difco) and the higher incubation temperature (27° versus 20°C) used in this baseline study. Nevertheless, the characteristic peak in bioluminescent activity was observed and was used as the end point in this study.

Effect of Acetone

The chemicals selected for testing were not readily soluble in an aqueous medium. A cursory comparison of the relative toxicity to R. harveyi revealed that acetone was less toxic than dimethylsulfoxide. Thus, a fixed volume of acetone was added to the salt solution to permit higher concentrations of nitrotoluenes and nitrobenzenes to be tested. A salt solution of 10% acetone was required to attain the desired range of concentrations of test

material in solution. Therefore, the effects of 0, 5, 10, 15, and 20% acetone salt solutions on the photobacteria were tested (Fig. 5). During the initial stages of incubation (ca. 7 hours), no differences among the five groups were detected. Thereafter, the five solutions eventually separated into three distinct groups. The 20% acetone solution produced the greatest level of response (decrease in peak luminescence), the 15% and 10% solutions formed the second group, and the 5% acetone solution showed no statistically significant difference ($p = 0.05$) from the salt solution control.

The 10% acetone solution did affect the time course of bioluminescent activity but was selected because this concentration of acetone was required to maintain the higher concentrations of the test chemicals in solution. Because a 10% acetone salt solution was used in all subsequent dose-response studies, any observed effects on bioluminescence must be qualified with regard to a potential interaction between the acetone effect and that of the test chemical.

Photobacterial Dose-Response Studies

Three dose-response studies were conducted on each of the nine test chemicals, and an EC50 was estimated for each curve. The mean and standard deviation of the three EC50 values were then computed. Table 3 lists the chemicals in order of decreasing potency as judged by their respective mean EC50 values. The EC50 values range from 3.45 to 20.25 mg/L and represent approximately a fivefold difference in potency. The estimates of the slope ranged from 13.61 to 115.24. The nine test chemicals showed the following order of potency based on their respective EC50 values: 2,5-dinitrotoluene (most potent); 2,3,6-trinitrotoluene; *m*-nitrobenzonitrile; 3,4-dinitrotoluene; *p*-nitrobenzonitrile; 2,4,6-trinitrotoluene; 2,3-dinitrotoluene; 2,4-dinitrotoluene and 2,6-dinitrotoluene (least potent).

There are no other published studies where B. harveyi has been challenged with these chemicals. Thus, no comparisons are possible. It may be concluded from this study that the intensity of the peak in bioluminescent activity (measured end point) is dose-dependent for the chemicals tested.

Comparison to Aquatic Toxicity Data. It is of interest to determine whether the photobacterial response is predictive of toxicity to other organisms. The fathead minnow, Pimephales promelas, and the water flea, Daphnia magna, are commonly used as indicators of aquatic toxicity. For each of the nine chemicals tested, a static 96-hour LC50 was determined in Pimephales promelas and a 48-hour EC50 in Daphnia magna. These data, first summarized by Pearson et al.,[31] are shown in Table 4. There was a fairly good correspondence of the relative toxicities of the nine chemicals between the fathead minnow and the water flea. The minnow's 96-hour LC50 also seemed to be the more sensitive index of toxicity. The only exception was *m*-nitrobenzonitrile where the 96-hour LC50 value in the minnow was greater than the 48-hour EC50 value in daphnia.

TABLE 3. SUMMARY OF DOSE-RESPONSE PARAMETERS IN R. HARVEYI

	Mean EC50 (± SD)[a]	Mean Slope
2,5-Dinitrotoluene	3.45 (0.40)	59.27
2,3,6-Trinitrotoluene	3.73 (0.25)	17.29
m-Nitrobenzonitrile	3.96 (1.36)	44.17
3,4-Dinitrotoluene	4.28 (0.38)	98.52
p-Nitrobenzonitrile	4.66 (0.65)	81.06
2,4,6-Tritnitrotoluene	6.54 (0.50)	13.61
2,3-Dinitrotoluene	7.67 (0.95)	115.24
2,4-Dinitrotoluene	8.26 (0.91)	64.10
2,6-Dinitrotoluene	20.25 (3.43)	111.39

a. Mean of three values expressed as mg/L.

TABLE 4. COMPARISON OF PHOTOBACTERIAL RESPONSE TO AQUATIC TOXICITY DATA

	Concentrations (mg/L)		
	Photobacteria EC50 (± SD)	Minnows 96-hr LC50	Daphnia 48-hr EC50
2,5-Dinitrotoluene	3.45 (0.40)	1.3	3.4
2,3,6-Trinitrotoluene	3.73 (0.25)	0.12	0.69
m-Nitrobenzonitrile	3.96 (1.36)	60.2	48.1
3,4-Dinitrotoluene	4.28 (0.38)	1.5	3.1
p-Nitrobenzonitrile	4.66 (0.65)	24.4	49.4
2,4,6-Trinitrotoluene	6.54 (0.50)	2.40	11.9
2,3-Dinitrotoluene	7.67 (0.95)	1.9	4.7
2,4-Dinitrotoluene	8.26 (0.91)	32.5	35.0
2,6-Dinitrotoluene	20.25 (3.43)	19.8	21.7

For three of the nine chemicals tested, the range of mean EC50 values observed in photobacteria was less than the range of LC50 values observed in minnows or EC50 values of the daphnia. Thus, in general, the photobacterial EC50 estimates appear to be less sensitive than the minnow 96-hour LC50 values. Furthermore, there was a poor correlation between the EC50 values from the photobacterial studies and the fathead minnow LC50 or the water flea EC50 values.

Interpreting the differences in "toxicity" between the photobacteria and the other two species is made even more difficult because of an organic solvent in the photobacterial test solution. None was used in the minnow or daphnia studies. As described earlier, acetone in the photobacterial test mixture produced a clear effect on the bioluminescence of B. harveyi. Thus, acetone in combination with one of the nitrotoluenes or nitrobenzonitriles may have resulted in either antagonism, additivity, or synergism. It would be of interest in future studies to determine if such interaction did occur under the conditions of this test.

In summary, the particular end point (EC50) used in this photobacterial test system does not seem to be very predictive of the acute toxicity of the same compounds in the minnow or daphnia. Perhaps the use of "no-effect" levels in the bioluminescent response of photobacteria may yield different conclusions.

Comparison to Acute Mammalian Toxicity Data. Mammalian toxicity studies become proportionately more costly and time consuming as the informational content derived from the conventional series of studies increases. Thus, there is a need for short-term screening tests predictive of mammalian toxicity. To evaluate the photobacterial system in this regard, the EC50 values were compared to the acute 14-day LD50 values in rats and mice for the same chemicals. The mammalian toxicity studies were conducted by Lee et al.[32] Table 5 presents the photobacterial and mammalian response data. First, male and female responses within each species differ. One sex is not consistently more susceptible to the acute toxic effects of the chemicals tested. There are instances of no differences in toxicity between male and female mice (2,5-DNT), situations where the males are clearly less sensitive (2,4-DNT), and times when the females are less sensitive (2,6-DNT). The substantial amount of variability in the extent of response even within a given species may make validation of the photobacterial response using such data more difficult.

The photobacterial response (EC50) was compared to the acute mammalian toxicity (LD50) by simply ranking the chemicals in order of relative toxicity in each system (Table 5). The ranking of LD50 values for male mice closely corresponded to the order of potency in photobacteria. The most toxic chemical in the male mouse was 2,5-DNT, which was also the most potent in the photobacterial system. Each chemical listed below 2,5-DNT in Table 5 shows a lesser degree of toxicity or potency. The exception was 2,6-DNT; the male mouse LD50 indicated it to be the most toxic of this group of chemicals,

TABLE 5. COMPARISON OF PHOTOBACTERIAL RESPONSE[a] TO ACUTE MAMMALIAN TOXICITY[b] DATA

	Photobacteria	Male Mice	Female Mice	Male Rats	Female Rats
2,5-Dinitrotoluene	3.45	652±28	659±12	616±34	517±25
3,4-Dinitrotoluene	4.28	859±37	747±26	907±42	807±33
2,4,6-Trinitrotoluene	6.54	1,014±52	1,009±54	1,010±41	820±32
2,3-Dinitrotoluene	7.67	1,372±34	1,089±32	1,102±20	911±65
2,4-Dinitrotoluene	8.26	1,954±68	1,340±67	568±59	650±49
2,6-Dinitrotoluene	20.25	621±51	807±35	535±58	795±22

a. Photobacterial response = Mean EC50 (mg/L).
b. Mammalian toxicity = LD50 ± SE (mg/kg).

whereas the photobacterial data indicate it to be the least toxic. This difference must be viewed in light of the relatively small range of the LD50 values for the chemicals studied. There is not much of a difference in the toxicity of 2,5-DNT as compared to 2,6-DNT.

The female mouse data indicate a lesser degree of correspondence to the photobacterial response but better than the rat data. A comparison of the rat LD50 values to the photobacteria EC50 values shows fairly good correspondence for four of the six chemicals listed in Table 5. This comparison is based on a ranking of the potency of each chemical in the rat and the photobacterial test system.

In summary, the influence of species and sex of the animal on the acute toxic response was evident in the data presented. For this reason, each sex of a given species was compared separately to the photobacterial response data. The highest degree of correspondence, determined by ranking the test chemicals in order of toxicity, was between the photobacteria and male mice. It should be noted again that the exception to the correspondence between the photobacterial response and the acute toxicity in male mice is an important one within the admittedly limited context of this study. It occurred with the chemical shown to be the most toxic in mice and the least toxic in photobacteria. It would be of interest to determine whether this relationship would continue if a larger number of related chemicals were tested. Equally important is the question of whether this photobacterial test system is predictive of the acute toxicity in male mice for other groups of chemicals such as the heavy metals.

Some limitations of this photobacterial test system must be described. First, chemicals not readily soluble in aqueous media will be difficult to test. If a response cannot be produced within the limits of the chemical's solubility, an organic solvent or colloidal suspension will be required for testing. Therefore, the results are difficult to interpret because of possible chemical-chemical as well as chemical-biologic interactions. Secondly, colored substances may mask the luminescent response of the photobacteria and thus reduce the system's applicability. A third possible source of problems is the testing of known bacterial mutagens. The photobacterial test system described in this study involves several replications of the bacterial test population. In the presence of a mutagen, it is conceivable that some of the bacteria may be transformed into "dark mutants."[5]

The nine chemicals used in this study are reported to be mutagens by Pearson et al.[32] This fact would have been important if bacterial population growth was observed without a concomitant increase in luminescence. It is interesting to extend this characteristic of the photobacteria and speculate on its potential usefulness as a mutagen screen. A dark mutant can be isolated in plate cultures and propagated. The exposure of dark mutants to chemical mutagens may then cause a reverse mutation to the light-producing type. The reverse mutation rate can very easily be quantitated by measuring the culture's luminescent activity as described in this study.

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APPENDIX A

Raw data with bioluminescence expressed as relative intensity. Readings during the first 3 hours of incubation resulted in no measurable bioluminescence.

TABLE A-1. EFFECT OF DIFFERENT ACETONE CONCENTRATIONS ON BIOLUMINESCENCE

Acetone (%)		Incubation Time (hours) 4	5	6	7	7.5	8	8.5	9	9.5
0		6	14	22	34	37	45	56	58	56
		5	11	20	34	38	47	54	65	57
		6	15	27	44	49	57	61	65	54
		6	15	28	43	48	54	58	58	52
		7	14	29	49	55	59	59	63	47
		6	13	26	41	46	54	58	61	51
	$\bar{x}$	6.0	13.7	25.3	40.8	45.5	52.7	57.7	61.7	52.8
	SD	0.6	1.5	3.6	5.9	6.9	5.5	2.4	3.2	3.7
5		6	14	26	42	47	56	58	63	52
		6	13	24	38	42	52	56	65	55
		6	15	28	43	47	56	58	63	51
		5	16	31	46	49	57	60	67	51
		7	14	32	49	53	59	56	57	45
		5	13	32	51	56	61	57	56	45
	$\bar{x}$	5.8	14.2	28.8	44.8	49.0	56.8	57.5	61.8	49.8
	SD	0.8	1.2	3.4	4.8	4.9	3.1	1.5	4.4	4.0
10		6	13	26	40	44	53	54	59	47
		6	14	28	44	48	58	55	59	46
		6	13	26	38	40	51	47	56	48
		7	15	28	44	47	55	49	50	38
		5	15	29	43	45	51	47	49	38
		5	15	30	47	50	57	51	49	39
	$\bar{x}$	5.8	14.2	27.8	42.7	45.7	54.2	50.5	53.7	42.7
	SD	0.8	1.0	1.6	3.2	3.5	3.0	3.5	4.9	4.8
15		5	13	26	39	43	52	50	52	40
		5	13	21	40	44	54	50	57	42
		5	13	26	40	44	54	49	56	44
		7	15	29	46	43	52	55	48	36
		7	15	26	41	43	52	55	48	36
		6	14	24	45	47	56	50	58	40
	$\bar{x}$	5.8	13.8	25.3	41.8	44.0	53.3	51.5	53.2	39.7
	SD	1.0	1.0	2.7	2.9	1.6	1.6	2.7	4.5	3.2
20		5	11	21	34	36	46	44	49	37
		5	10	21	33	35	44	41	50	38
		5	10	22	34	34	45	40	46	35
		7	12	25	38	38	47	40	47	35
		6	12	25	39	40	48	40	48	34
		6	13	27	42	42	50	39	43	31
	$\bar{x}$	5.7	11.3	23.5	36.7	37.5	46.7	40.7	47.2	35.0
	SD	0.8	1.2	2.5	3.6	3.1	2.2	1.8	2.5	2.5

TABLE A-2. EFFECT OF DIFFERENT STARTING VOLUMES ON BIOLUMINESCENCE

Volume Tested (µL)		Incubation Time (hours)								
		4	5	6	7	7.5	8	8.5	9	9.5
20		5	9	16	10	8	5	5	4	3
		5	9	16	10	7	5	5	4	3
		5	10	17	10	7	4	5	4	3
		5	9	16	11	8	5	4	4	2
		5	9	17	10	7	5	4	4	3
		5	9	16	11	8	5	5	3	3
	$\bar{x}$	5	9.2	16.3	10.3	7.5	4.8	4.7	3.8	2.8
	SD		0.4	0.5	0.5	0.6	0.4	0.5	0.4	0.4
40		5	9	16	24	16	10	10	8	8
		5	9	16	23	17	11	11	9	8
		5	10	17	21	14	9	9	8	7
		6	9	16	23	15	11	10	9	7
		5	9	17	24	15	11	10	8	8
		5	9	16	24	16	10	9	8	7
	$\bar{x}$	5.2	9.2	16.3	23.2	15.5	10.3	9.8	8.3	7.5
	SD	0.4	0.4	0.5	1.2	1.1	0.8	0.8	0.5	0.6
80		5	12	20	34	40	44	48	43	35
		5	12	21	36	43	48	51	47	39
		6	11	22	39	45	49	50	44	36
		5	12	23	40	47	50	53	48	39
		5	11	22	40	44	50	52	48	38
		5	11	22	40	47	48	52	48	37
	$\bar{x}$	5.2	11.5	21.7	38.2	44.3	48.2	51.0	46.3	37.3
	SD	0.4	0.6	1.0	2.6	2.7	2.2	1.8	2.3	1.6
100		6	13	24	40	47	59	63	66	60
		6	13	24	43	50	59	67	70	63
		6	14	25	44	51	55	68	72	64
		6	13	26	44	51	56	67	70	61
		6	14	25	45	53	57	68	70	60
		6	13	25	45	52	59	66	70	58
	$\bar{x}$	6.0	13.3	24.8	43.5	50.7	57.5	66.5	69.7	61.0
	SD	--	0.5	0.8	1.9	2.1	1.8	1.9	2.0	2.2
150		6	13	26	40	47	54	66	83	>100
		7	14	30	49	57	64	78	98	>100
		7	15	29	51	56	65	79	100	>100
		6	13	31	55	61	70	86	>100	>100
		6	14	30	53	59	69	84	>100	>100
		7	14	31	57	63	71	85	>100	>100
	$\bar{x}$	6.5	13.8	29.5	50.8	57.2	65.5	79.7	>100	>100
	SD	0.6	0.8	1.9	6.0	5.6	6.3	7.5	--	--
200		6	17	30	60	65	78	98	>100	>100
		7	14	30	54	63	76	89	>100	>100
		8	15	33	60	68	81	96	>100	>100
		7	17	33	61	69	82	97	>100	>100
		8	16	33	60	63	81	90	>100	>100
		8	15	32	53	70	85	98	>100	>100
	$\bar{x}$	7.3	15.7	31.8	58.0	66.3	80.5	94.7	>100	>100
	SD	0.8	1.2	1.5	3.5	3.1	3.2	4.1	--	--

APPENDIX B

Raw data from dose-response studies with bioluminescence expressed as relative intensity. Control group received salt solution.

TABLE B-1. RAW DATA FROM 2,3-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)				
		3.07	5.19	11.12	15.62	20.61
A	55	42	36	19	7	2
	55	42	35	19	7	3
	55	41	35	20	8	2
	53	41	36	18	9	3
	54	41	36	18	9	2
B	45	41	32	12	4	
	45	41	31	11	5	
	45	40	31	10	4	
	47	39	31	10	4	
	46	40	31	12	5	
	45	39	30	10	4	
C	43	41	36	20	8	1
	47	42	36	20	7	2
	46	42	36	19	7	1
	44	41	34	18	6	1
	50	43	36	19	5	1
	47	44	37	18	5	1

TABLE B-2. RAW DATA FROM p-NITROBENZONITRILE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)				
		1.94	3.77	7.54	14.61	20.88
A	49	36	25	12	3	0
	48	37	25	10	3	0
	47	37	25	10	2	0
	48	38	25	11	3	0
	49	38	26	12	3	0
B	46	39	25	13	6	2
	45	36	26	13	6	3
	45	37	27	13	6	3
	45	36	27	13	7	3
	46	37	26	14	7	3
	45	36	27	14	6	2
C	41	33	25	15	5	1
	39	33	26	14	5	1
	40	33	27	15	5	1
	39	33	26	16	6	1
	41	34	27	15	5	1
	41	34	27	15	6	1

TABLE B-3. RAW DATA FROM *m*-NITROBENZONITRILE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)					
		1.16	3.69	8.79	18.41	28.59	40.01
A	55	42	36	24	13	6	1
	57	43	35	25	12	6	1
	57	42	36	25	13	5	1
	55	42	35	25	12	6	1
	56	42	35	25	12	6	0
B	46	40	24	17	7	0	
	45	42	25	16	7	0	
	46	41	25	17	6	0	
	45	41	26	17	6	0	
	45	40	25	16	7	0	
	45	40	26	18	6	0	
C	41	31	26	16	11	5	2
	43	29	24	18	11	5	2
	45	30	24	17	10	5	1
	44	30	23	18	12	6	1
	44	29	24	18	11	6	2
	43	30	24	18	11	5	2

TABLE B-4. RAW DATA FROM 2,5-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)					
		2.36	4.10	11.78	17.31	24.22	32.82
A	54	38	24	11	5	3	1
	55	35	24	11	6	3	1
	53	35	25	10	5	2	1
	53	35	24	10	6	2	1
	54	35	25	11	6	3	1
B	36	24	13	5	2	0	
	36	24	14	5	2	0	
	37	23	13	5	2	0	
	39	24	13	5	2	0	
	38	23	13	6	3	0	
	37	23	13	6	2	0	
C	42	26	16	7	3	1	
	38	25	16	7	3	1	
	40	24	16	7	3	1	
	40	24	17	8	3	1	
	40	25	17	8	4	1	
	40	25	17	7	3	1	

TABLE B-5. RAW DATA FROM 2,4,6-TRINITROTOLUENE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)					
		0.96	2.56	4.71	6.54	7.72	9.00
A	57	55	47	40	27	15	9
	60	57	49	39	28	14	7
	58	56	50	41	27	15	8
	57	58	49	40	25	16	8
	61	58	50	38	28	15	7
	59	57	52	39	27	14	8
B	59	56	51	38	27	17	9
	59	60	51	39	25	16	11
	59	60	60	41	27	17	10
	61	59	57	43	29	18	12
	60	58	54	41	28	18	11
	61	60	58	40	32	21	10
C	66	60	59	49	37	25	18
	61	63	57	49	39	27	19
	67	62	60	50	39	26	18
	66	65	59	50	39	28	16
	65	65	59	51	38	28	18
	67	62	59	49	38	28	18

TABLE B-6. RAW DATA FROM 2,3,6-TRINITROTOLUENE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)				
		0.50	1.50	2.60	4.95	5.78
A	57	44	43	38	20	10
	55	41	44	39	24	10
	56	48	44	39	21	10
	62	44	42	37	21	9
	60	45	43	42	21	8
	59	47	44	38	23	13
B	59	50	45	42	20	9
	58	50	46	41	24	10
	60	52	49	46	22	11
	63	53	50	46	26	12
	64	55	52	46	27	13
	61	56	48	43	25	11
C	65	60	58	49	30	17
	63	61	58	50	29	15
	61	60	59	50	29	17
	63	62	56	47	29	16
	63	61	59	50	28	15
	61	62	58	49	32	18

TABLE B-7. RAW DATA FROM 3,4-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)				
		2.11	2.33	6.05	11.03	17.04
A	54	42	36	18	3	0
	54	41	37	18	2	0
	54	41	36	18	2	0
	52	41	36	17	2	0
	51	40	37	17	3	0
B	41	38	33	15	2	
	40	35	32	14	2	
	40	35	32	14	2	
	39	36	32	13	2	
	38	36	31	13	2	
	38	34	30	13	3	
C	41	34	31	18	6	1
	42	33	31	20	5	0
	44	32	31	22	6	1
	44	33	31	21	6	1
	47	34	33	20	6	1
	45	35	32	20	6	0

TABLE B-8. RAW DATA FROM 2,6-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)				
		9.01	13.77	19.21	23.20	27.00
A	53	42	33	25	19	14
	55	43	34	26	19	17
	57	44	33	24	20	15
	57	44	37	27	20	15
	55	44	38	26	18	15
B	35	38	28	23	18	
	35	35	30	22	18	
	36	36	29	22	18	
	35	36	31	21	18	
	38	35	30	23	19	
	37	37	29	23	18	
C	39	32	27	21	15	
	37	32	25	22	16	
	43	36	27	21	16	
	42	35	30	24	15	
	41	34	30	23	16	
	41	34	29	23	16	

TABLE B-9. RAW DATA FROM 2,4-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study	Control	Final Concentration (mg/L)				
		2.60	4.84	9.53	20.08	24.56
A	58	42	38	28	14	6
	57	43	37	27	14	6
	57	44	39	27	14	6
	58	45	39	29	13	6
	57	45	38	28	13	7
B	38	31	27	18	9	8
	36	31	27	18	9	7
	37	31	27	19	9	8
	37	32	25	18	10	7
	37	32	26	19	10	7
	36	32	25	19	10	7
C	35	28	23	17	9	
	35	27	23	16	8	
	36	28	20	16	9	
	37	27	21	16	8	
	36	29	24	17	9	
	36	29	24	17	9	

APPENDIX C

All data from dose-response studies were converted using the following formula:

Converted data = 100 - [(raw data/$\bar{x}$ of control group) x 100]

TABLE C-1. CONVERTED DATA FROM 2,3-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L) 3.07	5.19	11.12	15.62	20.61
A		22.8	33.8	65.1	87.1	96.3
		22.8	35.7	65.1	87.1	94.5
		24.6	35.7	63.2	85.3	96.3
		24.6	33.8	66.9	83.5	94.5
		24.6	33.8	66.9	83.5	96.3
	$\bar{x}$	23.88	34.56	65.44	85.30	95.58
	SD	0.99	1.04	1.54	1.80	0.99
B		9.9	29.7	73.6	91.2	
		9.9	31.9	75.8	89.0	
		12.1	31.9	78.0	91.2	
		14.3	31.9	78.0	91.2	
		12.1	31.9	73.6	89.0	
		14.3	34.1	78.0	91.2	
	$\bar{x}$	12.1	31.9	76.2	90.5	
	SD	2.0	1.4	2.2	1.1	
C		11.2	22.0	56.7	82.7	97.8
		9.0	22.0	56.7	84.8	95.7
		9.0	22.0	58.8	84.8	97.2
		11.2	26.4	61.0	87.0	97.2
		6.9	22.0	58.8	89.2	97.2
		4.7	19.9	61.0	89.2	97.2
	$\bar{x}$	8.7	22.4	58.8	86.3	97.1
	SD	2.5	2.1	1.9		0.7

TABLE C-2. CONVERTED DATA FROM *p*-NITROBENZONITRILE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L)				
		1.94	3.77	7.54	14.61	20.88
A		25.3	48.1	75.1	93.8	100
		23.2	48.1	79.3	93.8	100
		23.2	48.1	79.3	95.9	100
		21.2	48.1	77.2	93.8	100
		21.2	46.1	75.1	93.8	100
	$\bar{x}$	22.82	47.70	77.20	94.22	100
	SD	1.71	0.89	2.10	0.94	
B		14.0	44.8	71.3	86.8	95.6
		20.6	42.6	71.3	86.8	93.4
		18.4	40.4	71.3	86.8	93.4
		20.6	40.4	71.3	84.6	93.4
		18.4	42.6	69.1	84.6	93.4
		20.6	40.4	69.1	86.8	95.6
	$\bar{x}$	18.8	41.9	70.6	86.1	94.1
	SD	2.6	1.8	1.1	1.1	1.1
C		17.8	37.8	62.7	87.6	97.5
		17.8	35.3	65.1	87.6	97.5
		17.8	32.8	62.7	87.6	97.5
		17.8	35.3	60.2	85.1	97.5
		15.4	32.8	62.7	87.6	97.5
		15.4	32.8	62.7	85.1	97.5
	$\bar{x}$	17.0	34.5	62.7	86.8	97.5
	SD	1.2	2.0	1.6	1.3	

TABLE C-3. CONVERTED DATA FROM *m*-NITROBENZONITRILE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L)					
		1.16	3.69	8.79	18.41	28.59	40.01
A		25	35.7	57.1	76.8	89.3	98.2
		23.2	37.5	55.4	78.6	89.3	98.2
		25	35.7	55.4	76.8	91.1	98.2
		25	37.5	55.4	78.6	89.3	98.2
		25	37.5	55.4	78.6	89.3	100
	$\bar{x}$	24.64	36.78	55.74	77.88	89.66	98.56
	SD	0.80	0.99	0.76	0.99	0.80	0.80
B		34.7	60.2	72.9	88.9	100	
		33.2	58.6	72.9	90.5	100	
		33.2	58.6	72.9	88.9	100	
		34.7	61.8	72.9	88.9	100	
		37.9	60.2	72.9	88.9	100	
		33.2	58.6	71.4	88.9	100	
	$\bar{x}$	34.5	59.7	72.7	89.2	100	
	SD	1.8	1.3	0.6	0.7		
C		28.5	40.0	63.1	74.6	88.5	95.4
		33.1	44.6	58.5	74.6	88.5	95.4
		30.8	44.6	60.8	76.9	88.5	97.7
		30.8	46.9	58.5	72.3	86.2	97.7
		33.1	44.6	58.5	74.6	86.2	95.4
		30.8	44.6	58.5	74.6	88.5	95.4
	$\bar{x}$	31.2	44.2	60.0	74.6	87.7	96.2
	SD	1.7	2.3	1.9	1.5	1.2	1.2

TABLE C-4. CONVERTED DATA FROM 2,5-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L)					
		2.36	4.10	11.78	17.31	24.22	32.82
A		29.4	55.4	79.6	90.7	94.4	98.1
		34.9	55.4	79.6	88.8	94.4	98.1
		34.9	53.5	81.4	90.7	96.3	98.1
		34.9	55.4	81.4	88.8	96.3	98.1
		34.9	53.5	79.6	88.8	94.4	98.1
	$\bar{x}$	33.8	54.6	80.3	89.6	95.2	98.1
	SD	2.5	1.0	1.0	1.0	1.0	
B		35.4	65.0	86.5	94.6	100	
		35.4	62.3	86.5	94.6	100	
		38.1	65.0	86.5	94.6	100	
		35.4	65.0	86.5	94.6	100	
		38.1	65.0	83.9	91.9	100	
		38.1	65.0	83.9	94.6	100	
	$\bar{x}$	36.8	64.6	85.6	94.2	100	
	SD	1.5	1.0	1.3	1.1		
C		35.0	60.0	82.5	92.5	97.5	
		37.5	60.0	82.5	92.5	97.5	
		40.0	60.0	82.5	92.5	97.5	
		40.0	57.5	80.0	92.5	97.5	
		37.5	57.5	80.0	90.0	97.5	
		37.5	57.5	82.5	92.5	97.5	
	$\bar{x}$	37.9	58.8	81.7	92.1	97.5	
	SD	1.9	1.4	1.3	1.0		

TABLE C-5. CONVERTED DATA FROM 2,4,6-TRINITROTOLUENE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L) 0.96	2.56	4.71	6.54	7.72	9.00
A		6.3	19.9	31.8	54.0	74.4	84.7
		2.9	16.5	33.5	52.3	76.1	88.1
		4.6	14.8	30.1	54.0	74.4	86.4
		1.1	16.5	31.8	57.4	72.7	86.4
		1.1	14.8	35.2	52.3	74.4	88.1
		2.9	11.4	33.5	54.0	76.1	86.4
	$\bar{x}$	3.1	15.6	32.7	54.0	74.7	86.7
	SD	2.0	2.8	1.8	1.9	1.3	1.3
B		6.4	14.8	36.5	54.9	71.6	85.0
		0.3	14.8	34.8	58.2	73.3	81.6
		0.3	0.3	31.5	54.9	71.6	83.3
		1.4	4.7	28.1	51.5	69.9	79.9
		3.1	9.7	31.5	53.2	69.9	81.6
		0.3	3.1	33.1	46.5	64.9	83.3
	$\bar{x}$	1.7	7.8	32.6	53.2	70.2	82.5
	SD			2.9	4.0	2.9	1.8
C		8.2	9.7	25.0	43.4	61.7	72.5
		3.6	12.8	25.0	40.3	58.7	70.9
		5.1	8.2	23.5	40.3	61.2	72.5
		0.5	9.7	23.5	40.3	57.1	75.5
		0.5	9.7	21.9	41.8	57.1	72.5
		5.1	9.7	25.0	41.8	57.1	72.5
	$\bar{x}$	3.8	10.0	24.0	41.3	58.8	72.7
	SD	3.0	1.5	1.3	1.3	2.1	1.5

TABLE C-6. CONVERTED DATA FROM 2,3,6-TRINITROTOLUENE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L) 0.50	1.50	2.60	4.95	5.78
A		23.4	26.1	34.7	65.6	82.8
		29.5	24.4	33.0	58.7	82.8
		17.5	24.4	33.0	63.9	82.8
		24.4	27.8	36.4	63.9	84.5
		22.6	26.1	27.8	63.9	86.3
		19.2	24.4	34.7	60.5	77.7
	$\bar{x}$	22.8	25.5	33.2	62.8	82.8
	SD	4.2	1.4	3.0	2.6	2.9
B		17.8	26.0	31.0	67.1	85.2
		17.8	24.4	33.0	60.6	83.6
		14.5	19.5	24.4	63.8	81.9
		12.9	17.8	24.4	57.3	80.3
		9.6	14.5	24.4	55.6	78.6
		7.9	21.1	29.3	58.9	81.9
	$\bar{x}$	13.4	20.5	27.7	60.6	81.9
	SD	4.1	4.2	3.9	4.3	2.3
C		4.3	7.5	21.8	52.1	72.9
		2.6	7.5	20.2	53.7	76.2
		4.3	5.9	20.2	53.7	72.9
		1.1	10.6	25.0	53.7	74.5
		2.6	5.9	22.2	55.3	76.2
		1.1	7.5	21.8	48.9	71.3
	$\bar{x}$	2.6	7.5	21.6	52.9	74.0
	SD	1.4	1.8	1.9	2.2	2.0

TABLE C-7. CONVERTED DATA FROM 3,4-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L) 2.11	2.33	6.05	11.03	17.04
A		20.8	32.1	66.0	94.3	100
		22.6	30.2	66.0	96.2	100
		22.6	32.1	66.0	96.2	100
		22.6	32.1	67.9	96.2	100
		24.5	30.2	67.9	94.3	100
	$\bar{x}$	22.62	31.34	66.76	95.44	100
	SD	1.31	1.04	1.04	1.04	
B		3.4	16.1	61.9	94.9	100
		11.0	18.6	64.4	94.9	100
		11.0	18.6	64.4	94.9	100
		8.5	18.6	66.9	94.9	100
		8.5	21.2	66.9	94.9	100
		13.6	23.7	66.9	92.4	100
	$\bar{x}$	9.3	19.5	65.2	94.5	100
	SD	3.5	2.6	2.0	1.0	
C		22.4	29.3	58.9	86.3	97.7
		24.7	29.3	54.4	88.6	100
		27.0	29.3	49.8	86.3	97.7
		24.7	29.3	52.1	86.3	97.7
		22.4	24.7	54.4	86.3	97.7
		20.1	27.0	54.4	86.3	100
	$\bar{x}$	23.6	28.2	54.0	86.7	98.5
	SD	2.4	1.9	3.0	0.9	1.2

TABLE C-8. CONVERTED DATA FROM 2,6-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L) 9.01	13.77	19.21	23.20	27.00
A		24.2	40.4	54.9	65.7	74.7
		22.4	38.6	53.1	65.7	69.3
		20.6	40.4	56.7	63.9	72.9
		20.6	33.2	51.3	63.9	72.9
		20.6	31.4	53.1	67.5	72.9
	$\bar{x}$	21.7	36.8	53.8	65.3	72.5
	SD	1.6	4.2	2.1	1.5	2.0
B		-5.6	22.2	36.1	50.0	
		2.8	16.7	38.9	50.0	
		0	19.4	38.9	50.0	
		0	13.9	41.7	50.0	
		2.8	16.7	36.1	47.2	
		-2.8	19.4	36.1	50.0	
	$\bar{x}$	-0.5	18.1	38.0	49.5	
	SD	3.3	2.9	2.3	1.1	
C		21.0	33.3	48.1	63.0	
		21.0	38.3	45.7	60.5	
		11.1	33.3	48.1	60.5	
		13.6	25.9	40.7	63.0	
		16.0	25.9	43.2	60.5	
		16.0	28.4	43.2	60.5	
	$\bar{x}$	16.5	30.9	44.8	61.3	
	SD	4.0	5.0	3.0	1.3	

TABLE C-9. CONVERTED DATA FROM 2,4-DINITROTOLUENE DOSE-RESPONSE STUDIES

Study		Final Concentration (mg/L) 2.60	4.84	9.53	20.08	24.56
A		26.8	33.8	51.2	75.6	89.5
		25.1	35.5	53.0	75.6	89.5
		23.3	32.1	53.0	75.6	89.5
		21.6	32.1	49.5	77.4	89.5
		21.6	33.8	51.2	77.4	87.8
	$\bar{x}$	23.7	33.5	51.6	76.3	89.2
	SD	2.3	1.4	1.5	1.0	0.8
B		15.8	26.7	51.1	75.6	78.3
		15.8	26.7	51.1	75.6	81.0
		15.8	26.7	48.4	75.6	78.3
		13.1	32.1	51.1	72.8	81.0
		13.1	29.4	48.4	72.8	81.0
		1.31	32.1	48.4	72.8	81.0
	$\bar{x}$	14.5	29.0	50.0	74.2	80.1
	SD	1.5	2.7	1.5	1.5	1.4
C		21.9	35.8	52.6	74.9	
		24.6	35.8	55.3	77.7	
		21.9	44.2	55.3	74.9	
		24.6	41.4	55.3	77.7	
		19.1	33.0	52.6	74.9	
		19.1	33.0	52.6	74.9	
	$\bar{x}$	21.9	37.2	54.0	75.8	
	SD	2.5	4.6	1.5	1.5	

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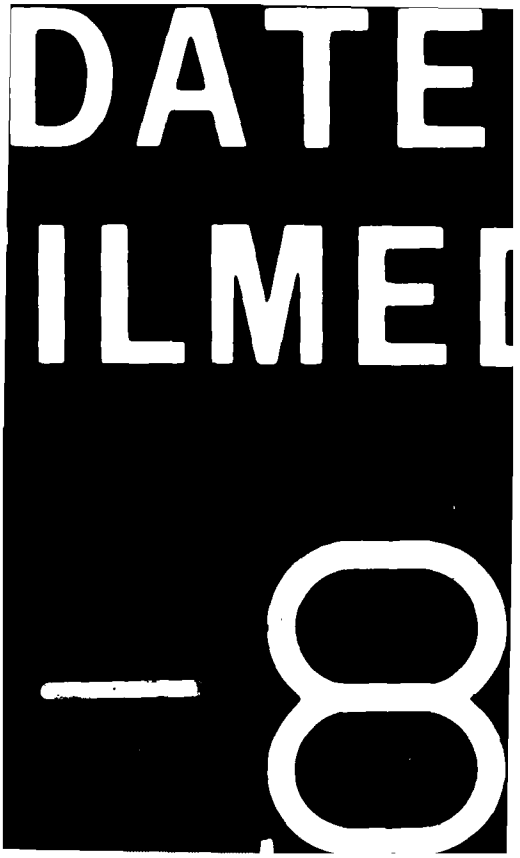
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